



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁶ : C12Q 1/68	A2	(11) International Publication Number: WO 98/40518 (43) International Publication Date: 17 September 1998 (17.09.98)
(21) International Application Number: PCT/US98/04819 (22) International Filing Date: 11 March 1998 (11.03.98) (30) Priority Data: 08/815,448 11 March 1997 (11.03.97) US (71) Applicant (for all designated States except US): WISCONSIN ALUMNI RESEARCH FOUNDATION [US/US]; 614 North Walnut Street, P.O. Box 7365, Madison, WI 53707-7365 (US). (72) Inventors; and (75) Inventors/Applicants (for US only): GUILFOYLE, Richard, A. [US/US]; 19912 Gateshead Circle, Germantown, MD 20876 (US). GUO, Zhen [CN/US]; Apartment 1044, 14611 N.E. 39th Street, Bellevue, WA 98007 (US). (74) Agent: BERSON, Bennett, J.; Quarles & Brady, P.O. Box 2113, Madison, WI 53701-2113 (US).		(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, GM, GW, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG). Published <i>Without international search report and to be republished upon receipt of that report.</i>
(54) Title: NUCLEIC ACID INDEXING (57) Abstract A restriction site indexing method for selectively amplifying any fragment generated by a Class II restriction enzyme includes adaptors specific to fragment ends containing adaptor indexing sequences complementary to fragment indexing sequences near the termini of fragments generated by Class II enzyme cleavage. A method for combinatorial indexing facilitates amplification of restriction fragments whose sequence is not known. Profiling methods and other methods for characterizing polynucleotides are presented.		

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Latvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav Republic of Macedonia	TM	Turkmenistan
BF	Burkina Faso	GR	Greece	ML	Mali	TR	Turkey
BG	Bulgaria	HU	Hungary	MN	Mongolia	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MR	Mauritania	UA	Ukraine
BR	Brazil	IL	Israel	MW	Malawi	UG	Uganda
BY	Belarus	IS	Iceland	MX	Mexico	US	United States of America
CA	Canada	IT	Italy	NE	Niger	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NL	Netherlands	VN	Viet Nam
CG	Congo	KE	Kenya	NO	Norway	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NZ	New Zealand	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's Republic of Korea	PL	Poland		
CM	Cameroon	KR	Republic of Korea	PT	Portugal		
CN	China	KZ	Kazakstan	RO	Romania		
CU	Cuba	LC	Saint Lucia	RU	Russian Federation		
CZ	Czech Republic	LI	Liechtenstein	SD	Sudan		
DE	Germany	LK	Sri Lanka	SE	Sweden		
DK	Denmark	LR	Liberia	SG	Singapore		
EE	Estonia						

NUCLEIC ACID INDEXING

CROSS-REFERENCE TO RELATED APPLICATIONS

10 This application claims priority to U.S. application serial number 08/815,448 filed March 11, 1997, which is incorporated herein by reference in its entirety.

BACKGROUND OF THE INVENTION

15 It is known in the art of molecular biology that a nucleic acid fragment lying between two identified and unique primer sequences can be amplified using the polymerase chain reaction (PCR) or modifications of the PCR. PCR avoids conventional molecular cloning techniques that require the existence in nucleic acid of advantageous restriction endonuclease cleavage sites. One identified shortcoming of PCR is that fragments
20 greater than about 40 kilobase pairs between the PCR primers are only weakly amplified. It has been difficult to obtain meaningful sequence data from large genomic fragments, particularly when such fragments are resistant to traditional cloning methods. Thus, the art is seeking new methods to
25 obtain the nucleic acid sequences of long, uncharacterized regions of genetic material.

Efforts to amplify a specific DNA cleavage fragment from a population of such fragments have included methods that involve cleaving the DNA using Class IIS enzymes or interrupted
30 palindrome enzymes to form fragments having non-specific terminal 5' or 3' overhangs of various lengths (generally 2 to 5 bases). Smith, D.R., PCR Methods and Applications 2:21-27, Cold Spring Harbor Laboratory Press (1992); Unrau, P. and K. Deugau, Gene 145:163-169 (1994); US Patent Number 5,508,169
35 (Deugau et al.); Zheleznaya, L.A. et al., Biochemistry (Moscow) 60:1037-1043 (1995). Class IIS enzymes cleave DNA asymmetrically at precise distances from their recognition

5 sequences. Interrupted palindrome ("IP") enzymes cleave
symmetrically between a pair of interrupted palindromic binding
sites. To amplify the products of such cleavages, nucleic acid
indexing linkers, containing protruding single strands
complementary to the cohesive ends of Class IIS- or IP cleavage
10 sites (rather than recognition sequences) and PCR primer sites,
have been annealed and ligated to fragments generated by Class
IIS- or IP cleavage.

The overhangs vary in base composition, and are determined
by the locations of the enzymes' cleavage sites in a genome.
15 The base composition and sequence of the overhang created after
cleavage with a Class IIS or IP enzyme cannot be predicted
because the sites at which those enzymes cleave DNA are
determined by spatial relationship to the recognition sequence,
but are not sequence-determined. In the methods described by
20 Smith, Unrau, Deugau and Zheleznaya, the unique cleavage sites
generated by Class IIS and IP enzymes determined a random
sequence by which fragments could be indexed. However, that is
not the case with more popular Class II enzymes that cleave
within their recognition sites and generate predictable,
25 identical sticky ends on each restriction fragment. Also,
Unrau's method employs temperatures that result in a problem of
illegitimate base pairing as well as problems with primer
dimers, where indexing fragments anneal with one another rather
with the target DNA.

30 What is desired is an indexing system that relies upon
fragments not generated by Class IIS or IP enzymes, and which
offer improved amplification specificity.

BRIEF SUMMARY OF THE INVENTION

The present invention is summarized in that
35 oligonucleotide adaptors for directing PCR amplification can be
engineered to efficiently and selectively hybridize "fragment
indexing sequences" of one or more bases immediately adjacent
to a Class II restriction enzyme recognition sites at the
termini of a nucleic acid fragment. A Class II enzyme cleaves
40 nucleic acid within its recognition site to generate a

5 characteristic 5' or 3' overhanging end or blunt end. The
recognition site can include one or more bases that do not form
part of the end that results from enzymatic cleavage. When the
adaptor and the nucleic acid fragment are brought together
under conditions suitable for intra-strand hybridization, the
10 invading strand of the adaptor displaces a portion of the
nucleic acid fragment.

Each oligonucleotide adaptor comprises a duplex portion
and a single-stranded portion. The duplex portion comprises an
invading strand and a complementary PCR primer strand
15 hybridized to the invading (displacing) strand. The
oligonucleotide adaptors for the two termini are distinct, in
that the PCR primer strands (and their complements on the
invading strand) of each end adaptor are selected to
specifically amplify fragments in the forward or reverse
20 direction. The PCR primer strand, which contains the sequence
that is the same as that used for a PCR primer, provides a 3'-
OH group that is required to join the adaptor to the
restriction fragment in the method. The invading strand, which
is longer than the PCR primer strand, also includes a
25 protruding single-stranded portion that comprises (1) a nucleic
acid sequence that can hybridize to the characteristic overhang
and (2) an adaptor indexing sequence that is perfectly
complementary to the fragment indexing sequence. The adaptor
indexing sequence is provided at the 5' end of the single-
30 stranded portion of the invading strand.

The invention is further summarized in that
oligonucleotide adaptors of the type described can be used in a
method for amplifying a restriction fragment that includes the
steps of:

- 35 (a) cleaving linear or circular nucleic acid at a
restriction enzyme recognition site with at least one rare-
cutting Class II restriction enzyme to generate a linear
restriction fragment having a characteristic 5' or 3' overhang
at each fragment terminus;
- 40 (b) hybridizing to each terminus of the fragment an end-
specific oligonucleotide adaptor, thereby displacing one strand

5 of the fragment;

(c) enzymatically ligating the restriction fragment to the primer strand to form a strand-displaced structure; and

(d) amplifying the strand-displaced structure.

10 The invention is further summarized in that a combinatorial degenerate mixture of oligonucleotide adaptors comprising every indexing sequence is also useful in a method for combinatorial indexing.

15 In a related aspect, the invention is summarized in that in a method for combinatorial indexing, genetic material cleaved with a rare-cutting enzyme produces a set of fragments for subsequent amplification. The cleaved DNA is added into an array of separate amplification reactions, where each reaction contains both an adaptor specific for one fragment indexing sequence and the degenerate combinatorial mixture of all
20 indexing adaptors specific to the other end of the fragment. Undesired complexity in reaction processing is avoided by including both the single end-specific adaptor and the combinatorial array of adaptors in the hybridization step.

25 In addition to obtaining valuable sequence data from the amplified fragments, it is possible to order the fragments by generating a restriction map by performing cross-digestion using two or more different enzyme arrays. By selecting the adaptor sequence, various PCR-related methods can be employed directly on the amplification products, including PCR
30 sequencing.

The invention is further summarized in that the adaptors are advantageously employed in methods for indexing, profiling, and characterizing polynucleotides. Adaptors can be grouped into desirable groups to acquire and analyze data gathered in
35 the indexing, profiling and characterizing methods.

It is an object of the present invention to facilitate genetic profiling.

40 It is another object of the present invention to facilitate accessing and sequencing regions of the human genome that are resistant to molecular cloning.

It is yet another object of the present invention to

5 amplify nucleic acid fragments with specificity.

It is a feature of the present invention that the overhang generated by cleavage with a Class II enzyme is predictable and invariant for each enzyme.

10 It is another feature of the present invention that the indexing sequence is separate from (not a part of) the overhang generated by restriction enzyme cleavage.

It is yet another feature of the present invention that a degenerate collection of adaptors containing all possible indexing sequences is used in combination with a defined
15 adaptor duplex to amplify unknown sequences of enzyme-cleaved nucleic acid.

It is an advantage of the present invention that the methods rely upon Class II enzymes rather than the less common Class IIS and IP enzymes.

20 It is another advantage of the present invention that the hybridizing regions of the fragments and adaptors are longer than have been used in previous indexing systems.

Another advantage of the present method is the remarkable specificity with which adaptors anneal to restriction fragments
25 when there is perfect matching between the bases of the indexing sequence and the complementary basis of the restriction fragment.

A fully automated PCR adaptor array strategy could bypass conventional cloning by simultaneously generating a restriction
30 map and DNA fragments for subcloning or direct sequencing from 0.5 Mb in about one day while avoiding problems associated with so-called unclonable regions. If large DNA pieces are to be mapped and sequenced, the DNA (up to about 0.5 Mb) must be purified using an existing technology such as site-specific
35 excision (RARE, achilles heel, PNA) or RARE-cutter restriction endonucleases (e.g., NotI or meganucleases (intron-encoded endonucleases)).

It is also possible to combine the method with conventional PCR, or to use the method in a process for
40 chromosome walking from the ends of fragments using indexers determined while preparing a restriction map.

5 Another application for the method is in genetic mapping to amplify fragments generated in restriction fragment length polymorphism (RFLP) analysis. Amplified fragments created from such fragments would be sequence-ready and could be used directly as probes in genetic mapping. It may also be
10 advantageous to first perform representational difference analysis (RDA) (Lisitsyn, N. et al. Science 259:946-951 (1993)) or RFLP-subtraction (Rosenberg, M. et al., PNAS USA 91:6113-6117 (1994)) to reduce the complexity.

The method could also be used as an alternative to AFLP
15 (Vos, P. et al., N. A. R. 21:4407-4414 (1995)) or arbitrarily-primed-PCR for analyzing altered gene expression by differential display (Perucho, M. et al., Methods in Enzymology 254:275 (1995); Liang, Methods in Enzymology 254:304 (1995)). This method would have advantages over AP-PCR such as reduced
20 noise and cleaner probes for gene hunting, better detection of rare messages, and a requirement for a smaller number of oligonucleotides.

Other objects, advantages, and features of the present invention will become apparent upon consideration of the
25 following detailed description taken in conjunction with the drawings.

BRIEF DESCRIPTION OF THE SEVERAL VIEWS OF THE DRAWINGS

Fig. 1 shows an embodiment of the restriction site indexing method of the present invention. The figure depicts
30 one end of a restriction fragment generated by cleavage with a Class II enzyme that generates a defined 5' overhang, a partially single stranded adaptor duplex and the displacement structure formed by hybridization and ligation of the fragment and the adaptor.

35 Fig. 2 shows a schematic embodiment of the invention where the restriction fragment generates a defined 3' overhang.

Fig. 3A depicts the end-specific adaptors used in the preferred embodiment to amplify the internal BclI fragments of
λ DNA.

40 Fig. 3B shows the degenerate set of combinatorial adaptors

5 used in the preferred embodiment to provide a proof of concept of the invention.

Fig. 4 shows the end-specific adaptors used in a method for differential display of cDNAs in accordance with the present invention.

10 Fig. 5 shows a strategy for amplifying 3' ends of mRNA (cDNA) using adaptors of the present invention.

DETAILED DESCRIPTION OF THE INVENTION

Reference is made to Fig. 1 which illustrates an embodiment of the restriction site indexing method of the present invention. In Fig. 1, a restriction fragment generated by cleavage with a Class II enzyme generates a defined 5' overhang (see left side of Fig. 1). In Fig. 2 (SEQ ID NO:27 through SEQ ID NO:31), a restriction fragment generated by cleavage with a Class II enzyme generates a defined 3' overhang (see left side of Fig. 2). When the enzyme generates a 3' overhang, the longer strand can act as both invading strand and primer strand. For example, in Fig. 2, the M13 forward primer (TGTAACGACGGCCAGT) (see also, SEQ ID NO:1) is the first 18 bases of the longer strand. The 18-mer primer oligonucleotide needs to be added for PCR amplification. No fill-in of the adaptor is required, as it is in the 5'-overhang case. Except as noted herein, the invention functions in the same manner when the enzyme generates a 3' overhang.

30 In the convention of this patent application, "forward" primers are specific for the "left" end of a fragment; "reverse" primers are specific for the "right" end of a fragment, where the fragment is presented with the 5' -> 3' strand as the top strand. As noted, a unique primer can be provided for all adaptors, if 2-strand sequencing is not desired.

35 Each fragment generated by cleavage of nucleic acid with a Class II restriction enzyme can be defined by a pair of fragment indexing sequences, defined as the one or more bases adjacent to the terminal recognition sites of a Class II restriction enzyme used to generate the fragment. Accordingly,

40

5 a unique pair of indexing adaptors, having the partially-singled stranded structures described herein, can hybridize to the two termini of a fragment.

Even though the characteristic overhangs at the termini are identical, the fragment indexing sequences adjacent to the recognition site are not predictable; any combination of bases
10 can reside at the indexing positions. It is noted that, because of an enzyme's cleavage strategy, one or more base pairs of the complete recognition site (e.g., in the exemplified embodiment of Fig. 1, an A-T pair) can remain near
15 the fragment terminus and should be accommodated during adaptor design.

Adjacent to the enzyme recognition site are the bases of the fragment indexing sequence, shown in Fig. 1 as X, which can be, but need not be, identical bases. In the fragment, Y
20 represents the base complementary to X at a given position. Thus, if X is A, Y can be T; if X is G, Y can be C; if X is C, Y can be G, and if X is T, Y can be A. Other recognized non-natural base pairs can also form. Because the fragment indexing sequence is not a part of the recognition or cleavage
25 sequence *per se*, neither the indexing sequence, nor its length, are limited by the choice of enzyme. This is an advantage over ligation-mediated indexing systems known in the art.

The chance that any one indexing sequence will correspond to more than one terminus decreases as the indexing sequence
30 length increases. Accordingly, it is desirable to select a preferred indexing sequence length. The suitable size of the fragment indexing sequence will depend upon the application to which the method is put. If the goal is specific fragment amplification, greater specificity is desired so the indexing
35 sequence should preferably be 3, 4, or 5 bases long. However, fragment fingerprinting or differential display of cDNAs can be accomplished using a preferable indexing sequence length of 1, 2, or 3 bases. An upper limit of 10 bases in the indexing sequence is contemplated.

40 By way of example only, the case of preparing adaptors for amplifying a fragment is considered. There are 64 3-base-long

5 indexing sequences, 256 4-base-long indexing sequences, and
1024 5-base-long indexing sequences. A 4-base-long indexing
sequence (256 choices) is preferred. Three- or five-base-long
indexing sequences could possibly be used, although if a
shorter sequence were used, the selectivity would be
10 compromised (in the sense that more fragments would be
amplified per adaptor pair), and if a longer sequence were
employed, sample handling becomes increasingly difficult
because of the array size.

It is also desirable to select a preferred nucleic acid
15 cleavage frequency. If many fragments are generated, the
likelihood that more than one fragment will be recognized by
identical adaptor pairs increases. One of ordinary skill will
appreciate that the desired number of fragments will depend
upon the application to which the method is put. If few
20 fragments are generated, PCR amplification of longer fragments
(with the accompanying art-recognized difficulties) will be
required.

Thus a rare-cutting enzyme is preferred. In methods for
restriction mapping or DNA fingerprinting, and for complex
25 genomes, the preferred restriction enzyme used to cleave the
target DNA is a 6-cutter. Five-cutters could be used, except
that they are few in number and recognize degenerate sequences,
thereby adding to the complexity of the required adaptors.
Four-cutters are thought to be unsuitable because of their
30 abundant distribution of cleavage sites. Enzymes cutting at
sites of greater than 6 bases are also believed to be
unfeasible, given their extreme rarity in the genome. On the
other hand, for genomes of lower complexity, or for RNA
fingerprinting (using cDNA targets) and differential display
35 applications, 4-cutter enzymes would be suitable. Combinations
of enzymes having different cleavage frequencies can be well
suited for generating fragments having a certain desired
average size, or for a particular target sequence.

A simple calculation for 6-cutters predicts that 256
40 individual, sequence-ready restriction fragments can be
amplified from a target DNA of up to 0.5 megabases (Mb) in

5 size. DNA of 1 Mb complexity digested with a 6-cutter enzyme,
which cleaves a random sequence on average every 4096 base
pairs, will produce 244 fragments, on average. Dividing this
by 256 indexers yields about 1 amplified fragment per end-
specific adaptor/combinatorial adaptor pair used. An indexing
10 sequence would be present twice in the full library (array) of
adaptors, with one contributed by the end-specific adaptor and
the second by the combinatorial adaptor. A fragment would be
amplified twice, but at different locations in the array, and
therefore a 0.5 Mb target DNA segment would be accommodated
15 bidirectionally for isolating individually amplified
restriction fragments. If the target DNA is greater than 0.5
Mb, the method is still applicable using either complete
digests or partial random digests such that more than one
restriction fragment may be amplified per well.

20 The above-noted combination furnishes the convenience of
easy to automate arrays of 256 members and a distribution of
restriction sites that yields amplification lengths compatible
with state-of-the-art PCR amplification technology.

The center of Fig. 1 shows an indexing adaptor of the type
25 described. Indexing adaptors contain a region for PCR priming
(or other function), a region complementary to a Class II
restriction enzyme recognition site, and a strand-displacement
region which is complementary to the fragment indexing sequence
adjacent to the recognition site on the overhang strand.

30 Although it is referred to herein for convenience as the
PCR primer strand, the strand can comprise any sequence that is
desired to be placed at a terminus of a fragment having the
specified indexing sequence and can provide any desired
function, for example, a restriction enzyme recognition /
35 cleavage site, to facilitate subsequent processing of amplified
fragments. Thus, the adaptors of the present invention have
appreciably broader utility than for PCR amplification. If the
function to be provided by the adaptor is PCR amplification,
then the sequence should be unique or present in low copy
40 number, should provide an available 3' end and should be
recognized by a suitable polymerase enzyme, such as Taq or TthI

5 polymerase. The -21M13 forward primer or the M13revP reverse primer (together, "the M13 primers") are suitable primers if the amplified fragments will be used for subsequent bi-directional sequencing. The -21M13 and M13revP primers are specific for the left and right ends of a restriction fragment,
10 as those terms are used herein. The M13 primers, used as described herein, permit amplified fragments to be sequenced on both strands. If bi-directional sequencing is not desired, distinct primers need not be provided. For terminal fragments of linear nucleic acid molecules, a suitable amplicon-specific
15 terminal primer can be provided in place of an adaptor if the terminal sequence is known. The sequences for amplifying the fragment can also be sequences for elongation of a template by a DNA or RNA polymerase, such as a T3 promoter, a T7 promoter, an SP6 promoter, or a sequence complementary to same.

20 The invading strand includes a portion complementary to the primer strand. Also, adjacent to that portion is a sequence that can hybridize to the Class II enzyme recognition site of the fragment terminus (including any residual bases near the fragment terminus that do not form part of the
25 overhang) to form the displacement structure shown at the right in Fig. 1. Note that although a second displacement structure, wherein the indexing sequence is displaced by the restriction fragment, could form, it is not favored and is not observed, for it results in a net loss of 5 nucleotides available for
30 annealing by the invading strand.

Strand-displaced structures of this type are described in EP-A-0 450 370 A1, Quartin et al., Biochemistry 28:8676-8682 (1989), Weinstock and Wetmur, Nucleic Acids Res. 18:4207-4213 (1990), and Wong et al., Nucleic Acids Res. 19:2251-2259
35 (1991), which are incorporated herein by reference in their entirety, most particularly the parts relating to the formation, structure and properties of strand-displaced structures useful in the present invention.

The above-noted documents describe Branch Capture
40 Reactions ("BCR") that involve sequence-dependent attachment of a single-stranded tail to a duplex DNA, in which one strand of

5 the duplex is displaced by the single-stranded tail. The
strand-displaced structure formed in BCR is akin to that of the
present invention, and the parameters of formations described
in the publications may be used in carrying out indexing
methods of the present invention. However, the documents are
10 directed to capturing specific, individual DNA fragments from a
mixture using unique sequences rather than a mixture of
indexing sequences, and concern direct cloning of the captured
fragments rather than indexing analysis of polynucleotides in a
mixture.

15 The documents also relate that formation of strand-
displaced structures and capture of specific fragments can be
facilitated by incorporating duplex-stabilizing modified bases
in the capture tail. The present invention can be carried out
without using modified bases in the adaptor single-stranded
20 indexing region. Nevertheless, modified bases can be employed
in the present invention to stabilize duplexes formed between
an indexing adaptor and a polynucleotide to be indexed. In
some circumstances, it may prove particularly advantageous to
do so. Among the modified bases of this type are pyrimidines
25 substituted with bromine at C5, particularly C5-substituted
BrdC (5-bromodeoxycytidine). Also useful in this regard are 5-
methyl substituted pyrimidines, particularly 5-
methyldeoxycytidine.

DNA ligase efficiently joins the adaptor to the
30 restriction fragment only if the adaptor indexing sequence is
perfectly complementary to the corresponding fragment indexing
sequence. Even one mismatched base in the adaptor indexing
sequence will discourage efficient ligation and subsequent PCR
amplification relative to a perfectly matched adaptor.

35 However, the hybridizing portion need not be completely
complementary to the overhang, in the sense of classic Watson-
Crick base pairing. A universal mismatch base analog (such as
3-nitropyrrole) could be positioned within the restriction site

5 to elicit an effect on the indexing sequence moiety. Moreover,
a string of such base analogs could be used to completely
replace every base within the restriction site, so that all
four indexer bases could experience enhanced discrimination and
a universal adaptor could be developed for most 6-cutter
10 restriction enzymes. This would require that the base analog
or analogs incorporated not greatly affect ligase activity.

By positioning a universal base mismatch in 3 to 4 base
proximity to a natural base mismatch, the T_m is lowered by up
to 8°C relative to a perfect match. This discrimination
15 enables one to amplify only fragments that perfectly match the
indexing sequence provided from a digest containing many
fragments. Although this can lower overall duplex stability by
as much as 15°C, the enhanced discrimination would be
significant for the indexing sequences. This is because
20 discrimination is generally reduced at natural base mismatches
near 3' ends, for example, where the indexer sequences are
located in the adaptor oligonucleotides.

Both positional and compositional differences may have an
effect upon hybridization efficiency. It is anticipated that
25 differences in discrimination by adaptors for indexing
sequences may relate to GC content, illegitimate base pairing
issues, proximity to the site of ligase joining, and contiguous
base stacking effects.

One or more natural base analogs (such as 5-nitroindole)
30 can also be added to the overhanging 5' end of an adaptor, if
desired, to center the indexing sequence in the hybridizing
portion thereby further enhance discrimination between exact
and mismatched indexing sequences. The number of such bases
that can be added can be as long as the number of bases in the
35 portion of the invading strand that is complementary to the
restriction recognition sequence.

Improved discrimination is most apparent when the
universal mismatch nucleotide is provided in either of the
first two positions adjacent to the indexing sequence unless
40 the position is itself adjacent to a mismatch, which causes
reduced stability. When the universal mismatch is provided any

5 closer than three bases from the site at which subsequent ligation occurs, it is thought that the non-natural base interferes with ligation efficiency and less amplified product is produced relative to that amount produced after combining the adaptor having a perfectly matched indexing sequence.

10 The indexing adaptor can be formed by hybridizing a primer strand and an invading strand together under standard annealing conditions. A primer strand and an invading strand can be synthesized separately using oligonucleotide synthesis methods that are conventional in the art. Many oligonucleotide primers
15 for use as primer strands are readily commercially available. The M13 primers are commercially available, are in widespread use, and can be fluorescently tagged. In addition, the M13 primers have annealing temperatures that are very close to one another. This property is desirable in that both the forward
20 and reverse amplifications can proceed with comparable efficiency under a single set of conditions. As noted, the two sequencing primers need not be used if direct sequencing is not desired.

The invention can be embodied in a method for amplifying
25 fragments of known sequence, using readily engineered adaptors having suitable adaptor indexing sequences specific for both ends of the known fragment. Also, by providing combinatorial mixtures comprising all possible adaptors specific to the fragment ends, one can amplify any fragment without knowing the
30 identity of the indexing sequence specific for either terminus. The invention can also be practiced on a fragment where one end is known but the other end is unknown, by employing in the method one end-specific adaptor or amplicon-specific primer for the known fragment end and a combinatorial adaptor mixture for
35 the other fragment end thereby permitting amplification of a fragment containing known and unknown sequences, such as intron regions and flanking sequences beyond viral junctions.

The method is applicable to various targets including
40 previously "unclonable" regions from genomic DNA, since there is no need to clone such fragments to obtain useful DNA sequence. Also, large fragments can be directly cleaved and

5 isolated from complex genomes for subsequent analysis using the
method. Also, intron sequences, the sequences flanking viral
integrants, can be isolated and sequenced, as can terminal
fragments from YAC, BAC, P1, plasmid or cosmid clones. The
method can also be used to generate STS-like probes at rare-
10 cutter restriction sites. Also, it will be possible to excise
fragments surrounding regions of ambiguous sequence for further
sequencing using the method.

In a method embodying the present invention, a population
of fragments is generated from a nucleic acid sample by
15 cleaving the sample with a Class II restriction enzyme. The
identity of the Class II restriction enzyme is not critical,
except to the extent that the sequence of the terminal overhang
must be known, for preparing suitable adaptors. A
comprehensive list of restriction endonucleases, including
20 Class II enzymes, in Robert and Macelis, Nucleic Acids Research
26: 338-350 (1998); see also <http://www.neb.com/REBASE> on the
world wide web. When selecting a restriction enzyme and
designing the respective adaptors for use with that enzyme for
restriction mapping or isolation of "sequence-ready" fragments,
25 it is advantageous, but not essential, to minimize the
differences in the composition of the recognition site by
forming an overhang whose 4 bases are G, A, T and C. Any of
about 50 known Class II 6-cutters (including isoschizomers)
generate 3' or 5' overhangs whose 4 bases are G, A, T and C.
30 The available enzymes include, but are not limited to, BamHI,
HindIII, AvrII, ApaLI, KpnI, SphI, NsiI, and SacI. Among these
enzymes, only the outermost base remaining after cleavage will
vary in composition. The outermost base makes only a small and
almost inconsequential contribution to the T_m for adaptor-
35 fragment annealing. This facilitates the ligation protocol,
but is not to be considered essential to the invention. This
design parameter also facilitates the method by helping to
confine discrimination analysis to the base composition of the
indexer sequences. In addition to Class II enzymes that
40 generate four base overhangs, other enzymes that may be used
effectively in the method are those that cleave palindromic

5 sequences in opposite polarity, those that leave either blunt ends or different length overhangs (e.g., not 4-base overhangs), and those that leave base compositions other than A, G, T, and C.

10 After cleavage, one or more pairs of partially single-stranded indexing adaptors are hybridized under standard annealing conditions to the termini of one or more fragments generated by the enzyme cleavage. Each fragment can hybridize to a single pair of adaptors. As noted above, the sequence that complements the restriction recognition sequence can
15 include an universal mismatch to improve discrimination between adaptor indexing sequences that are perfectly-matched and imperfectly-matched to the fragment indexing sequences. *Bona fide* amplification occurs when adaptors containing perfectly-matched indexing sequences are hybridized, thus there is
20 advantage to favoring the ability of such sequences to hybridize. Hybridization should be sufficiently strong to permit subsequent ligation of fragment termini to a pair of adaptors.

After hybridization, the gap between the primer strand and
25 the overhanging strand of the restriction fragment is closed by treating the structure with DNA ligase under standard conditions (see Fig. 1, right side), thereby joining the overhanging strand to the primer strand. T4 ligase (NEB), thermostable Ampligase (Epicentre Technologies) ligase enzymes
30 are suitable and have been used successfully at temperatures up to 50°C. Other ligases may also be used. Suitable ligation conditions are typical of those used in the art. The result of this step is to introduce an end-specific PCR primer (or other desired sequence) onto each end of each fragment. The primer
35 is attached only to fragments bearing a suitable indexing sequence.

Note that during hybridization the single-stranded portion of the adaptor hybridizes to its complementary sequence on the overhang strand and displaces the fragment indexing sequence
40 (and any residual bases of the recognition site) on the opposite strand. In the special case of a 5' terminal

5 overhanging fragment (shown in Fig. 1), the invading strand is not covalently joined to the restriction fragment. Thus, before amplification can proceed, the displaced strand is extended from its 3'-end by polymerase in the first thermal cycle to regenerate a template complementary to the PCR primer.
10 This extension step is not required if the termini have 3' overhangs (Fig. 2).

Fragments can be amplified using standard PCR reactions such as those described in the Example. In the preferred embodiment, one set of PCR conditions is suitable to amplify
15 fragments of most sizes, although it may be necessary in certain cases to adjust the PCR conditions in accordance with the abilities of one skilled in the art to amplify a particular fragment. PCR protocols can be varied to accommodate particular sequences and primers. One skilled in the art will
20 appreciate that certain modifications to the PCR protocols may be required to amplify particular fragments. Such modifications may include varying primer length, adjusting magnesium concentration, adjusting thermal cycle time, adjusting the annealing temperature and the like. It is
25 necessary to add additional primer before amplifying. One skilled in the art will also appreciate, for example, that so-called long distance PCR conditions can be employed to amplify fragments greater than about 3 kb, although success under such conditions cannot be assured, as such protocols are still under
30 development by the art.

Occasional false amplifications may be observed if a particular indexing sequence forms a more stable mismatch when hybridizing with the restriction fragment. However, one having ordinary skill can determine hybridization conditions under
35 which such mismatches are not observed and do not give rise to amplification products.

In another aspect, the invention is also a system for combinatorial indexing. Combinatorial indexing is advantageously employed when seeking to separately amplify
40 restriction fragments where the index sequence of each fragment terminus is not known. It will be appreciated that by

5 providing every adaptor specific to both ends, all fragments
generated by enzyme cleavage can be amplified, even without a
priori knowledge of the sequence. In the method described
above, by contrast, each fragment terminus has an indexing
sequence selected from one of the possible indexing sequences
10 (e.g., 1 of 256 possible 4-base-long indexing sequences). The
unique combination of indexing sequences corresponding to the
termini of an unknown fragment is one of 65,536 possible
pairwise combinations of 256 left-end-specific indexing
sequences and 256 right-end-specific indexing sequences.

15 Such a large array of possible combinations is
methodologically impractical (even if automated), but would be
necessary to recover all possible restriction fragments that
could be generated from total digestion of a larger DNA. Even
if automated, the handling of such a large array would be
20 formidable. However, the size of the array can be reduced to
256 simply by providing in each reaction a single unique left
or right end-specific adaptor along with a degenerate mixture
of 256 adaptors corresponding to the second fragment end. Such
mixtures are referred to herein as a "combinatorial adaptor" or
25 a "C-adaptor." The C-adaptor mixture can be made in a single
oligodeoxynucleotide synthesis process by providing all 4
nucleotides (A, G, C, T) at each adaptor indexing sequence
position.

Reducing base-pairing specificity provides a way to
30 control the number of possibilities, by combining adaptors into
sets or by substituting modified bases that can pair with more
than one base, or both. For instance, Py- and Pu-specific
bases would have a 1 in 2 probability of pairing with each base
in a random sequence, whereas any of A, C, G and T have a 1 in
35 4 possibility. Consequently, for $n=2$, for example, using any
of A, C, G or T for position 1 and either Py or Pu for position
2 produces only 8 possibilities rather than 16. Likewise, for
 $n=3$, using Py or Pu in place of A, C, G or T for any one of the
three positions provides 32 instead of 64 "sequences." The
40 ability to control the number of indexing sequences in this way
may be used to reduce the number of reactions and separations

5 needed to index a given polynucleotide population.

The specificity at each position of an indexing sequence may be provided by A, C, G, T or modified bases. Reduced specificity may be provided by mixing indexing sequences or by synthesizing oligonucleotides with two or more bases in one or
10 positions of the indexing sequences. Thus, N can be provided by mixing indexing sequence having each of A, C, G and T at the given position of N in the indexing sequence, or it can be provided by synthesizing an oligonucleotide with a mixture of A, C, G and T at the given position for N. N-specific
15 positions can also be provided using modified bases (such as those described elsewhere herein).

A PCR reaction would yield an amplified fragment only when it contains both the end-specific indexing sequence as well as to one of the indexing sequences in the combinatorial adaptor.
20 In 256 separate ligation/PCR reactions, the probability is that each reaction amplifies a single, sequence-ready restriction fragment. Although the invention is practiced by providing an adaptor specific to each end when 2-strand direct sequencing of the PCR products is desired, the invention can also be
25 practiced by providing a single primer for both ends. The invention can also be practiced using a single adaptor, if PCR amplification is not desired. For example, a restriction fragment and a primer strand tagged with a reporter molecule can be annealed to a surface-bound invading strand, without
30 subsequent ligation. The restriction fragment will anneal to the invading strand where there is correspondence between the adaptor- and fragment indexing sequences. The primer strand will also anneal to the invading strand. After annealing, unbound restriction fragments can be washed away. Interstrand
35 base stacking interactions between the tagged primer strand and the restriction fragment will keep the primer strand annealed only where the fragment corresponds to the invading strand indexing sequence. This can facilitate specific detection of restriction fragments of interest. When used in this manner,
40 the invention provides a method for ordering fragments in a clone.

5 To map the order of fragments, several independent arrays are analyzed as described using adaptors specific for different restriction enzymes and then the product of each array can be cross-digested with the enzymes of the other digestions. The products of those cross-digestions can be separated by
10 electrophoresis and a standard restriction map can be produced for any nucleic acid fragment.

Ligation-mediated indexing using class-II enzymes can be applied to RNA fingerprinting in a way similar to that described for class-IIS enzymes (Kato, K. NAR, 24:394-395
15 (1996), incorporated herein by reference). A particular application in this regard would be for functional identification of genes by differential cDNA display. Kato and others proposed that an indexing approach could offer several advantages over the more commonly used "arbitrarily primed PCR"
20 (Liang, P. and Pardee, A.B. Science, 257:967-971 (1992), incorporated herein by reference) for this purpose, including (a) obtaining more coding regions, (b) allowing lower redundancy, and (c) detecting rare messages more efficiently.

An important aspect of such a fingerprinting application
25 is the ability to adequately resolve the fragments generated. For example, differentiated or neoplastic somatic cells have a messenger RNA complexity on the order of 20×10^6 . Using a pair of 4-cutter restriction enzymes to digest cDNA, fragments are obtained that should, on average, be <200 bp in size. A
30 given message will be represented by numerous non-overlapping fragments specifically amplified using adaptors with 4-nucleotide indexing sequences. The fingerprint of the 256 fragment subclasses generated can be well resolved on a polyacrylamide gel.

35 The order of the fragments for a given message can be determined either by (a) restriction mapping and/or sequencing the clone(s) from an appropriate cDNA library that cross-hybridize to the amplified fragments, or (b) amplifying the cDNA using the identified message-specific indexing adaptors in
40 conjunction with primers which can access the 5'- and/or 3'-end of the message, and then restriction mapping and/or sequencing.

5 As examples, the 5'-end of an mRNA can be located after preparing the cDNA using CapFinder technology (Clontech); the 3'-end of an mRNA can be accessed using oligo-dT primers as described by Liang and Pardee or oligo-dT coupled with a different or universal primer.

10 Single-enzyme strategies could also be used to obtain RNA fingerprints using indexers for class-II enzymes. Indexing can be confined to one of the cleaving enzymes if the second cleaving enzyme generates a constant, defined end. These strategies would target either the 5'-proximal or 3'-proximal
15 restriction fragments of the cDNA. The cDNA could be cut with a single 4-cutter, ligated to the indexing adaptors containing a universal primer, and then PCR amplified by using either a CapFinder or oligo-dT associated primer. These approaches would yield less complex fingerprints than the double-enzyme
20 approach, but would be biased toward detecting fewer coding regions and more untranslated regions (UTRs). However, UTRs represent excellent signatures for identifying unique messages.

Different strategies could be adopted to reduce array size and, therefore, sample handling. One strategy could utilize
25 the combinatorial adaptors. Instead of using 256 single-end adaptors, adaptors could be pooled in several combinatorial mixtures which represent subclasses of the complete library (e.g. 4 pools x 64; 16 pools x 16, etc.). (A pooled subclass could also be synthesized as a degenerate oligo). The
30 complexity of the banding pattern (per pool) will decrease as the number of pools increases. In another strategy, 3-nucleotide indexing sequences could be utilized. The size of a 3-nucleotide indexing sequence library would be 64. However, because trinucleotide frequencies are higher than
35 tetranucleotide frequencies in a given genome, a more complex banding pattern is expected.

Another application for the method is genetic profiling, including DNA fingerprinting and RNA fingerprinting. A particularly useful DNA fingerprinting application would be
40 detecting restriction fragment length polymorphisms. These RFLPs detect polymorphic sequences distributed throughout a

5 genome and serve as useful markers for genetic linkage mapping.

Traditional RFLP analysis required hybridizing probes of known sequence to genomic DNA digested with various restriction enzymes. However, newer methods do not require any prior probe characterizations and can be applied to the
10 fingerprinting of genomes of any complexity. These newer methods include random amplified polymorphic DNA (RAPD), DNA amplification fingerprinting (DAF), arbitrarily-primed PCR (AP-PCR), and amplified fragment length polymorphism (AFLP). In each of these methods, except AFLP, random genomic DNA
15 fragments are amplified using by arbitrarily selected primers to generate fragment patterns for any DNA without prior knowledge of its sequence. AFLP (Vos, P. et al., N.A.R. 21:4407-4414 (1995)) resembles RFLP (Bostein, D. et al., Am. J. Hum. Genet. 32: 314-331), except insofar as restriction
20 fragments are detected by PCR rather than Southern hybridization. Also, AFLP displays the presence or absence of fragments rather than their size differences. The adaptors that are ligated to the digested DNA in AFLP analysis include sets of generic PCR primers, thereby permitting the sequences
25 which reside adjacent to the restriction sites to be queried. In AFLP, fingerprints of varying complexity can be obtained by adjusting the enzymes and primer sets employed.

AFLP can also be used for RNA fingerprinting to detect and monitor differential gene expression, starting with different
30 double-stranded cDNA samples for a given comparative analysis (Money, T., et al. N.A.R. 24:2616-2617). Regardless of the source of DNA, however, a major disadvantage of this method is that it requires many variations in adaptor and/or PCR primer designs. This, in turn, demands that PCR conditions be
35 optimized for each selected set of primers. Therefore, the AFLP technique is not highly amenable to formatting for streamlined multi-sample processing.

In the present invention, as in the AFLP method, fragments are queried at sequences located next to their sites. In the
40 case of the present invention, however, an adaptor invading strand, rather than PCR primers, is used to interrogate those

5 sequences. The adaptors can contain combinations or
permutations of indexing sequences that anneal by
strand-displacement to their corresponding polynucleotides.
Indexing adaptors of the present invention can contain one
primer sequence (pair) for amplifying the polynucleotide after
10 the adaptor is ligated. Therefore, only one set of PCR
conditions need be found for all fragments amplified, without
regard to the enzyme or indexing sequences employed.
Furthermore, the indexing specificity is relatively insensitive
to changes in temperature, time, and ligase concentration
15 conditions used in ligating the adaptors to the polynucleotides
(results not shown). This is not unexpected since it is known
that the annealing reaction during branch migration at the
termini of fragments is very rapid and efficient (Quartin,
R.S., et al., Biochemistry 28:8676-8682).

20 Taken together, these advantages mean that no variations
in adaptor design or PCR conditions is intrinsically necessary,
unlike in AFLP, making the method highly amenable automation
and high throughput.

Genetic profiling using the present invention can be
25 carried out using a variety of strategies, for both DNA and RNA
fingerprinting. For RFLP analysis, profiles would be expected
to show only the presence of new fragments or the absence of
fragments resulting from mutations or sequence variations in a
restriction fragment and/or an indexing sequence. The
30 fragments created to score RFLPs would be generated using
restriction enzymes that cut at frequencies amenable to PCR
amplification and display by gel electrophoresis.

An RFLP strategy is chosen based on the complexity of the
genomic DNA interrogated as well as the desired complexity of
35 the fingerprint. As in the AFLP method, important variables
include the character of class-II enzymes used (e.g., 4-cutter,
6-cutter) and the indexing sequencing length(e.g., 2, 3, or 4
nucleotides). For both RNA and DNA profiling, a given approach
could utilize mixtures of strand-displacing adaptors and
40 sticky-end adaptors, such as is used in an RNA fingerprinting
approach for displaying differentially-expressed sequences that

5 represent full length messages (see Examples). Alternatively,
a single enzyme-adaptor set could be ligated to bring in only
one PCR primer, the other being provided by a known sequence
located elsewhere in the restriction fragment. This
approach was used for the gene expression profiling of
10 restriction fragments derived from the 3'-ends of mRNA in a
manner resembling that described by Prashar and Weismann (U.S.
Patent No. 5,712,126, incorporated herein by reference in its
entirety; see also PNAS USA 93: 659-663 (1996) and see
Examples).

15 Unlike the widely used arbitrarily primed PCR method
(Perucho, M. et al., Methods in Enzymol. 254: 275 (1995) and
Liang, Methods in Enzymol. 254:304 (1995), the present
invention is a form of "ligation-mediated PCR" that more
efficiently detects low-abundance messages by significantly
20 reducing the redundancy of amplified products. This is so
because arbitrarily selected primers randomly amplify cDNA
sequences in their entirety whereas in a ligation-mediated
approach, a single pair of primers (brought in by adaptors)
amplify specific portions of a message.

25 Adaptor primer sequences can serve least four purposes:
(1) amplifying by PCR to generate profiles, (2) re-amplifying
specific bands by PCR for isolating and subcloning, (3)
re-amplifying specific bands for use as a direct sequencing
template, and (4) generating DNA or RNA probes by PCR or by *in*
30 *vitro* transcription, respectively. A variety of strategies can
be employed for designing adaptor primer sequences. For PCR
applications, the primer sequences are preferably designed such
that their T_m 's closely match one another, and so that the
primer lengths accommodate the type of PCR reaction employed.
35 Longer sequences may be desired, for example, to enable
two-step thermal cycling, touchdown PCR, or long-distance PCR
conditions. In certain cases, some sequences, such as the T7,
T3, and SP6 promoter sequences, could be used for all four
applications.

40 It may be desirable to have more than one primer sequence
per adaptor, to accommodate a custom designed utility for more

5 than one function. Resulting increases in adaptor size are not
expected to significantly change the efficiency of ligation to
the restriction fragments. Using primer sequences built into
the adaptors, fragments isolated from fingerprints of genomic
DNA or mRNA can be re-amplified and sequenced to serve as
10 sources of genetic mapping probes or "expressed sequence tags"
(EST), respectively. For DNA profiling, it may be advantageous
to first perform a subtractive technique to reduce complexity,
such as representational difference analysis (RDA) (Lisitsyn,
N. et al., Science 259: 946-951 (1993) or "RFLP-subtraction"
15 (Rosenberg, M. et al., PNAS USA 91:6113-6117 (1994)). For RNA
profiling, it may also be advantageous to first perform a
subtractive technique to enrich for differentially expressed
genes (for example, see Ariazi, e. and Gould, M. J. Biol.
Chem., 271:29286-29294 (1996)).

20 A targeted amplification strategy similar to that employed
by U.S. Patent No. 5,712,126 (Prashar and Weissman),
incorporated herein by reference in its entirety, can also be
used to amplify 3'-end restriction fragments of cDNAs to
generate "fingerprints" or "expression profiles" from which
25 bands can be recovered for sequence analysis and EST
production. These fragments contain mostly untranslated
sequences, which can serve as unique identifiers for messenger
RNAs. These sequences are also useful for creating and
searching EST databases.

30 By using strand-displacement adaptors in conjunction with
enzymes that cut relatively frequently (e.g., 4-cutter class-II
enzymes), the present invention will achieve significantly
greater gene coverage than can be obtained with the Prashar and
Weissman technology, which typically employs 6-cutter class-II
35 enzymes. While U.S. Patent No. 5,712,126 can require up to 55
6-cutters to obtain >95% gene coverage for 3'-fragments up to
400 bp in size, it is expected that >99.9% gene coverage can be
obtained using only four 4-cutters in combination with
tetranucleotide indexing sequences.

40 The strategy to generate expression profiles and ESTs in
this manner is as follows:

5 (1) make double-stranded cDNA from total RNA or polyA+ RNA using anchored oligo-dT/heel sequence;

(2) digest cDNA with a 4-cutter to produce polynucleotide fragments;

10 (3) ligate fragments to strand-displacement adaptor containing restriction site and indexing sequence;

(4) PCR amplify fragments using primers complementary to adaptor and anchored heel sequence, where one primer is distinguishable, e.g., includes a radiolabel, fluorescent dye label, or infrared dye label;

15 (5) separate the amplification products, e.g., on a denaturing polyacrylamide gel;

(6) detect the distinguishable products by, for example, autoradiography (for radioisotopic labeling), fluorimaging (for fluorescent dye labelling) or IR-imaging (for infrared dye labelling);

20 (7) excise bands of interest (with optional re-amplification step), and determine their nucleic acid sequences; and

(8) search databases and analyze sequences.

25 Genes that are differentially expressed can be profiled and recovered by using the above strategy on samples representing different cellular states such as (a) normal vs. diseased, (b) infected vs. uninfected, (c) developing vs. adult, (d) drug treated vs. untreated, and the like. Profiles are preferably displayed by running PCR products side-by-side
30 on a denaturing polyacrylamide gel to readily observe fragments that represent genes of unchanged or altered expression. The profiling aspect of the invention can be advantageously employed in a search for novel pharmaceuticals that, for
35 example, promote or inhibit mRNA expression by cells in a particular state. In particular, characteristic reference, average or diagnostic profiles can be established for sets of cells that exhibit differential mRNA expression.

40 The most stable linkages between adaptors and fragments will likely be obtained using restriction endonucleases that

5 generate the longest possible overhangs (Weinstock, *supra*). In the case of 4-cutters, these would be tetranucleotide overhangs such as those generated by Sau3AI (DpnII) and Tsp509 I for 5'-overhangs, and Tai I and Cha I for 3'-overhangs.

Examples

10 Amplification

The feasibility of the specific amplification method described herein was tested using N⁶-methyladenine-free bacteriophage λ DNA (48502 base pairs, New England Biolabs, Beverly, MA) as the model amplicon system and BclI, a 6-cutter, as the model Class II restriction endonuclease. Enzyme digestions were performed in the supplier's buffer at 37 °C for two hours with 20 U of BclI in a volume of 100 μ l. BclI cuts the λ genome eight times, producing nine fragments that share the same 5'-overhang sequence, 5'-GATC. BclI was chosen because of the broad range of fragment sizes that the enzyme generates: 517, 560, 1576, 2684, 4459, 4623, 6330, 8844, and 18909 base pairs. The terminal fragments are 560 and 8844 base pairs. The terminal fragments include a BclI cut site at one end and the genome terminus at the other. Unique oligonucleotide primers were used to amplify the terminal λ fragments.

Since the entire nucleic acid sequence of the λ genome is known, adaptors were produced containing only the required adaptor indexing sequences. In the adaptors, the primer strand was either an M13 sequencing primer or M13 reverse sequencing primer, depending upon which end of the fragment it was specific for. Terminal primers were provided for the terminal fragments. The invading strand comprised, in 5' to 3' order, a 4-base-long indexing sequence, a 5-base-long sequence complementary to the BclI recognition site, and a portion fully and perfectly complementary to the primer strand. The primer strand and the invading strand were prepared by conventional oligonucleotide synthesis, were purified on Sep-Pak C18 cartridges and were annealed at a concentration of 12.8 μ M of each primer in 50 mM tris-HCl, pH 7.8 at 85°C. The

5 oligonucleotides were allowed to anneal by slow cooling to room temperature.

The end-specific indexing sequences used to amplify particular λ BclI fragments are shown in Fig. 3A (SEQ ID NO:1 through SEQ ID NO:20). The end-specific adaptors that
10 corresponded to the left (L) and right (R) ends of the fragments used the -21M13 (forward) and M13RevP (reverse) universal primer sequences, respectively. For each end, the primer strand is shown once and each partially-complementary end-specific invading strand is shown. The indexing sequences
15 specific to each fragment end are shown in bold and the BclI site that remains after cleavage is underlined.

Once the adaptors were prepared, the BclI fragments were individually amplified from the total BclI digest as follows:

(a) 5 μ g of N⁶-methyladenine-free λ DNA (New England
20 Biolabs, Beverly, MA) was digested at 37°C for 2 hours with 20 units of BclI in a volume of 100 μ l using the manufacturer's (NEB) buffer;

(b) 15 ng of digested λ DNA were combined with left and right adaptor pairs corresponding to a particular restriction
25 fragment in NEB 1X ligase buffer for 5 minutes at 40°C (each ligation contained 25 pmols of single end adaptor pairs, in equal amounts. For the right end of the genome, λ -specific primer CGTAACCTGTCGGATCAC (SEQ ID NO:21) was used. To amplify the left end of the genome (8848L), λ -specific oligonucleotide
30 CGCGGGTTTTCGCTATTT (SEQ ID NO:22) was used);

(c) 800 units of NEB T4 DNA ligase were added and the reactions were incubated for 20 minutes at 40°C and were stopped by heating to 65°C for 15 minutes;

(d) 1.5 ng of λ DNA were transferred to 100 μ l PCR
35 reactions. All PCR reactions were performed with the XL-PCR kit (Perkin-Elmer, Applied Biosystems Division, Foster City, CA), using 2 μ l (4 units) of rTth DNA polymerase. The PCR reactions included 1.1 mM magnesium acetate (1 mM MgCl₂ carried over from the ligase reaction), except the amplification of the
40 4,459 base pair BclI fragment from λ DNA which included 1.65 μ l of magnesium acetate to obtain robust and specific

5 amplification from its combinatorial adaptor. The specific products could also be obtained using 0.55 mM magnesium acetate. All PCR reactions contained 10 pmols of appropriate primer oligonucleotides. PCR was performed in the PTC-200 DNA
10 cycling profile: 95°C for 1.5 minutes followed by 30 cycles of 94°C for 40 seconds, 55°C for 40 seconds, 72°C for 5 minutes. Treatment with 3'-to-5' exonuclease activity of Vent polymerase was important for increasing the yields of the PCR products obtained with rTth polymerase.

15 (e) 20 μ l were loaded on 0.8% agarose gels containing 0.5 μ g per μ l ethidium bromide. Specific bands were observed upon electrophoresis.

No reactant removal or product purifications were required between steps, making the overall procedure amenable to
20 automation. In some conditions, it may be advantageous, but not absolutely necessary, to purify fragment-bound adaptors away from unligated adaptors or fragments. A solid-phase purification step can be included. However, the need for such a solid-phase purification has not been observed.

25 When the appropriate left/right adaptor pairs or terminal/left or right adaptor pairs were used, eight of the nine BclI fragments of λ DNA were selectively and specifically amplified. Under the conditions described, specific amplification of the 18909 base pair fragment was not observed,
30 although the fragment was observed with additional non-specific fragments, including the 6.3K, 4.6K, 4.4K, 2.6K and 1.5K lambda fragments. These fragments were amplified at least in part because a longer polymerase extension time was required just to detect the 18909 base fragment. In this case, fragments
35 arising from rare non-specific ligation events are amplified to a greater extent. However, when 3-nitropyrrole was incorporated into the restriction site of the adaptor, all of the extra bands were eliminated. The suppression of the nonspecific fragments was optimal when the 3-nitropyrrole was
40 positioned in the middle of the 9-nucleotide protruding single-strand region of each of the 18K-specific adaptors.

5 It is possible to achieve good discrimination among the
adaptor pairs tested. Where non-targeted restriction fragments
were co-amplified along with the desired product, the extra
amplification can be explained by homology in some indexing
10 sequence positions and the potential for stable mis-match
duplex formation in other indexing sequence positions. Few
non-specific products that did not co-migrate with the
restriction fragments were observed.

Combinatorial indexing

15 To demonstrate the utility of the method employing
combinatorial adaptors, two sets of combinatorial primers were
prepared, as is shown in Fig. 3B. The "combo-FP" adaptor
included the -21M13 primer hybridized to the indicated C-
adaptors, where N at each position indicated in the adaptor
represents a population of all four nucleotides at that
20 position. Thus, each mixture of combinatorial adaptors
included 256 different adaptors. Likewise, the "combo-RP"
adaptor set included the M13revP primer hybridized to the
indicated set of invading strands where N is all four
nucleotides at each position.

25 To amplify various fragments of BclI-cut λ DNA, the
following amounts of the indicated end-specific adaptors (or
primers in the case of the terminal fragments) were combined
with the indicated amounts of combo-FP or combo-RP mixtures.

Table I

30	Fragment to be amplified (bp)	Right adaptors (pmol)	Left adaptors (pmol)	Combo-FP mix (pmol)	Combo-RP mix (pmol)
	517				
	560	10 (560R*)	---	0.0025	---
35	1576	25	---	0.5	---
	2684	25	---	0.25	---
	4459	25	---	25	---
	4623	25 pmol	---	25	---
	6330	---	---	---	---
40	8848	---	10 (8848L*)	---	0.0025

*Primer only (in PCR reaction)

5 Specific amplification of fragments having the expected
fragment length were observed by polyacrylamide gel
electrophoresis, thus indicating that desired fragments can be
amplified by providing an adaptor specific for one end of a
10 desired fragment and a mixture of adaptors containing an
adaptor specific for the indexing sequence at the other end of
the fragment. It is of note that no purification was required
prior to PCR amplification to remove ligation reactants or
intermediate products.

15 Specific fragment amplification was driven predominantly
by the end-specific adaptor ligated at one end. That is
because when the end-specific adaptor and C-adaptors are
provided at equimolar amounts, the relative concentration of a
single indexing sequence in the combinatorial mixture is only
1/256 as great as the amount of the end-specific adaptor,
20 thereby favoring more efficient ligation of the more prevalent
adaptor.

 In additional tests, it was shown that specific fragments
were amplified from the total BclI- λ DNA digest over a range of
asymmetric end-specific:C-adaptor concentration ratios. The
25 ratios of end-specific adaptors:C-adaptors was varied from 1:1
to 100:1. An additional hundred-fold dilution of the
combinatorial adaptor yielded the most specific λ terminal
fragment amplifications.

Amplification from genomic polynucleotides

30 To demonstrate that specific amplification can be
accomplished in the presence of a more complex genome, *E. coli*
DNA containing λ c1857Sam7dam⁻ lysogen (NEB) was used as the
amplification target. This more complex genome (4.7 Mb) has
1,604 BclI sites, 200 times as many as λ DNA. Despite this
35 increase in target complexity, λ BclI fragments could still be
specifically amplified using the adaptors tested.

 Eighteen μ g of the λ lysogen DNA was digested with BclI.
Twenty five pmol (each) of left and right adaptors were used to
amplify the 517, 1576, and 2684 bp fragments. Subsequent
40 dilutions and reactions were performed as described above for λ

5 DNA.

Although the concept has been demonstrated using known DNA, it is equally applicable to unknown DNA targets excised directly from the genome. Using the method, a DNA fragment that maps between two STS markers can be obtained. At least
10 two 6-cutter arrays will be used in conjunction with combinatorial indexing to obtain a complete restriction map of the selected fragment and the production of contigs. PCR amplification products produced from each array will be subjected to agarose gel electrophoresis to acquire fragment
15 length information.

RNA fingerprinting

RNA fingerprinting using adaptors for class-II enzymes was tested for the differential display of cDNA from rat mammary carcinomas, untreated or treated with perillyl alcohol (PA)
20 which is a monoterpene used for chemoprevention and chemotherapy (Crowell, P.L. and Gould, M.N. Crit. Rev. Oncog., 5L:1-22 (1994), incorporated herein by reference). cDNA from treated and untreated tumors (at half-regression) was prepared by and according to Ariazi, E. and Gould, M. (J. Biol. Chem.,
25 271:29286-29294 (1996), incorporated herein by reference).

In a preliminary study, DpnII (GATC) and NlaIII (CATG) were used as the cleavage enzymes. DpnII provides indexing sequences next to its 5'-overhang and NlaIII provides a defined 3'-overhang for a cohesive end adaptor. Because a DpnII site
30 will not anneal with an NlaIII site, fragment chimeras are minimized and primer-dimer formation during PCR is eliminated. As is shown in Figure 4, the NlaIII adaptor contains the M13 reverse primer sequence and the DpnII adaptors contain the M13 forward primer sequence. For this study, four 4-nucleotide
35 indexing sequences were used (Fig. 4, SEQ ID NO:1 and SEQ ID NO:23 through SEQ ID NO:28). The adaptors were designed such that the chance of forming stable mismatches was minimized according to the observations of Ebel et al., Biochemistry 31:12083-12086 (1992), incorporated herein by reference.

5 A suitable protocol for generating fingerprints was as follows. Note that if the enzyme cleavage buffers are compatible with one another, the cleavages can be accomplished in a double digestion.

- (1) digest 0.5 μ g cDNA (-/+ PA treatment) with NlaIII;
 - 10 (2) clean-up*, elute in water;
 - (3) join NlaIII adaptor (25 pmol) with 800U T4 DNA ligase at 37°C;
 - (4) clean-up, elute in water;
 - (5) digest with Dpn II;
 - 15 (6) clean-up, elute in water;
 - (7) split cDNA four ways (125 ng ea.) and join Dpn II adaptors (25 pmol) with 800U T4 DNA ligase at 40°C;
 - (8) use KlenTag (Advantage cDNA PCR kit, Clontech, Palo Alto, CA) to amplify 5 ng of ligated DNA using 25 pmol ea. of the -21M13 and M13rev primers;
 - 20 (9) run aliquots on 5% polyacrylamide electrophoresis gels; stain with Sybr Green I (Molecular Probes, Eugene, OR) to separate and visualize a characteristic pattern for amplified fragments;
 - 25 (10) visualize by UV transillumination or laser scanning (Fluorimager 575, Molecular Dynamics, Sunnyvale, CA)
- * each clean-up step used Qiaquick spin column (Qiagen, Chatsworth, CA) to remove enzymes, buffers and/or unligated adaptors

30 For the two 4-cutter approach, an average expected number of amplified products per gel lane (i.e. per indexer) was estimated by $(20 \times 106/512)/256$, or approximately 150, assuming a perfectly random distribution of sites and a perfectly random sequence of nucleotides in the total cDNA. However, because

35 the sequences are not random in nature, fragment size range varies. For the 4 indexing adaptors tested, the size of the observed amplified fragments ranged from about 50 bp to about 300 - 500 bp. The bands were well separated and indicated a quasi-random distribution of restriction sites useful for

40 fingerprinting and probe isolations. The fingerprints observed were highly reproducible for a given set of thermal cycling

5 parameters and yielded differentially expressed bands
indicating both up-regulation and down-regulation after PA
treatment (confirmed by varying the amount of template in the
PCR). The sensitivity of the assay was high, detecting as
10 little as 2-3 fold changes in the levels of some differentially
expressed bands. However, to distinguish truly differentially
expressed bands from false positives, it would typically be
necessary to re-amplify a band and use it as a probe against
Northern blots.

Amplification of mRNA/cDNA 3' ends

15 The 3'-end-targeted amplification strategy employing the
adaptors of the present invention was tested on Sau3AI digested
cDNA prepared from resting and activated human (Jurkat)
T-lymphocytes. Activated Jurkat T cells are known to contain
highly elevated levels of interleukin-2 (IL-2) mRNA. A 40-mer
20 oligonucleotide (CAGGGTAGACGACGCTACGC(T₁₈)AT; SEQ ID NO:32) was
used as an anchor primer for cDNA synthesis and PCR primer in
the fragment amplification step. In SEQ ID NO:32,
CAGGGTAGACGACGCTACGC is the "heel" sequence, T18 is an 18-mer
oligo-dT portion that can anneal to the poly-A tail of
25 messenger RNAs, and AT is the dinucleotide anchor sequence. AT
was chosen because its complement is contained in interleukin-2
(IL-2) mRNA. The 3'-proximal Sau3AI site of IL-2 cDNA is
located 142 bp from the poly-A. The indexing sequence adjacent
to the 3'-proximal Sau3AI site is AAAA on the anti-sense strand
30 of the restriction fragment.

An adaptor was prepared by annealing an 18-mer
oligonucleotide (TGTAACGACGGCCAGT; SEQ ID NO:1) corresponding
to the M13 forward primer sequence and a 26-mer invading strand
that contained the M13 sequence complementary to the forward
35 primer sequence, the Sau3AI tetranucleotide overhang and the
AAAA indexing sequence. Fig. 5 depicts the strand displacement
structure formed by ligating the adaptor to the IL-2 Sau3AI
fragment as well as the primers used for PCR. Fig. 5 also
shows eight indexing sequences tested (IS-1 through IS-8)
40 besides that for IL-2 (IS-9). An IL-2 specific fragment of 200

5 bp is amplified (26 bp +40 bp + 142 bp - 8 bp, where 8 bp is the overlap between adaptor and fragment).

Total RNA was prepared, cDNA was synthesized, adaptors were formed, polynucleotides were digested, ligated and amplified using Ampligase Gold (ABI-Perkin Elmer) as described
10 in U.S. Patent 5,712,126, except that the restriction enzyme and adaptors of Prashar and Weissman were substituted by Sau3AI and the indexing adaptors, respectively. For detecting the amplified fragments, the M13 forward primer was end-labeled with ³²P using polynucleotide kinase according to a standard
15 protocol. Amplified restriction fragments were separated by electrophoresis on an 8M urea-6% polyacrylamide denaturing gel, and an autoradiograph was obtained by exposing the dried gel to X-ray film for approximately 16 hours.

The nine fingerprints observed on the autoradiograph are
20 non-overlapping (i.e., share no apparent bands) and contain fragments representing differentially expressed genes. Base-pairing specificity within the indexing sequence was determined by re-amplifying, subcloning and sequencing five fragments excised from each of the nine fingerprints. No base pair
25 mismatches were observed, indicating 100% specificity for 40 fragments that were targeted (5 fragments did not yield readable sequence in this experiment). Less than 20% non-specificity was observed in the systemic background, that is only in non-targeted cDNA fragments revealed only in the
30 fragment library subclones. Close to the number of expected fragments (see table below) were observed, strongly suggesting that gene coverage efficiency is high under the ligation and PCR reaction employed.

Furthermore, the fingerprints were consistently
35 reproducible with respect to band patterns and signals, and identical fingerprints were observed by fluorescent imaging when a tetramethylrhodamine (TAMRA)-labeled M13 primer was substituted for the ³²P-labeled M13 primer. From sixty nonredundant sequences analyzed from the nine adaptor profiles,
40 34 matches with human genes were found in the Genbank-primate database (5 differentially expressed, including IL-2), 22

5 matches in the gb-EST database were found (2 differentially
expressed), and four fragments showed no matches (1
differentially expressed) and represent novel genes.
In the preceding example, simple, well-resolved fingerprints
(15-20 fragments per lane) are generated by using a
10 dinucleotide anchor primer in combination with tetranucleotide
indexing sequences. Although this combination yields close to
the theoretical number of expected 3'-fragments (~10), the
number of ligation reactions to achieved 99% gene coverage is
extremely large, and for 4 enzymes is [256 adaptors] x [12
15 N₁N₂-oligo(dT) primers] x 4 = 10,240. This would be a
formidable task even if automated.

Table 2
Parsing Reduction Strategies

		5'-end		3'-end	# expected frags/lane	# ligations (4-enzs)
20	Strategy	IS	length	set		
	I	4	256	N1N2 (x12)	10	10,240
	II			N1 (x 3)	40	2,560
	III			N0	120	853
25	IV	3	64	N1N2	40	3,072
	V			N1	160	768
	VI			N0	480	256

Table 2 shows six parsing reduction strategies that could be
employed to reduce the number of reactions to a manageable
30 size. These strategies do not consider the use of
"combinatorial mixtures" of base-pairing specificity, which could
further reduce the total number of reactions required. In
Table 2, parsing reduction can be targeted to one or both ends
of the restriction fragments by reducing the number of indexing
35 bases (5'-end) from four to three in the ligation step, or
oligo-dT anchor bases (3'-end) from two (N₁N₂) to one (N₁) to
zero (N₀) in the PCR step. Table 2 shows the reduction in the
number of reactions for the six different 5'/3' combinations,
with a proportional increase in the number of expected
40 fragments per lane. In the case of N₀, only the "heel" portion
(see Fig. 5) need provide the sequence for a 3' PCR primer.

5 These latter numbers were statistically derived using 4-cutter
sites of sequences from the Expressed Gene Anatomy Database
(EGAD) of The Institute for Genomic Research (www.tigr.org) and
projected for mRNA complexity of 15,000 unique transcripts.
Only 3'-proximal fragments up to 400 bp in size were considered
10 in the statistical analysis since this represents the upper
limit of gel resolution. This size, on the other hand, is
significantly greater than the mean size of fragments generated
by 4-cutters and therefore only a small fraction will be lost
by gel exclusion.

15 From Table 2, it can be seen that strategies III&V are
likely candidates for parsing reduction since they yield
manageable numbers of both reactions and resolvable fragments.
Strategies I, II and IV produce too many reactions, while
strategy VI produces a large number of fragments that cannot be
20 readily resolved.

 The present invention is not intended to be limited to the
preceding embodiments, but rather to encompass all such
variations and modifications as come within the scope of the
appended claims.

5

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: Guilfoyle, Richard A
Guo, Zhen

(ii) TITLE OF INVENTION: Nucleic Acid Indexing

10 (iii) NUMBER OF SEQUENCES: 32

(iv) CORRESPONDENCE ADDRESS:

15 (A) ADDRESSEE: Quarles & Brady
(B) STREET: 1 South Pinckney St.
(C) CITY: Madison
(D) STATE: WI
(E) COUNTRY: US
(F) ZIP: 53703

(v) COMPUTER READABLE FORM:

20 (A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30

(vi) CURRENT APPLICATION DATA:

25 (A) APPLICATION NUMBER:
(B) FILING DATE:
(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:

30 (A) NAME: Berson, Bennett J
(B) REGISTRATION NUMBER: 37094
(C) REFERENCE/DOCKET NUMBER: 960296.94053

(ix) TELECOMMUNICATION INFORMATION:

(A) TELEPHONE: 608-251-5000
(B) TELEFAX: 608-251-9166

35 (2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:

40 (A) LENGTH: 18 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(A) DESCRIPTION: /desc = "-21M13 forward primer"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

TGTAAACGA CGGCCAGT

18

5 (2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

- 10 (A) LENGTH: 27 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

- (A) DESCRIPTION: /desc = "end specific
adaptor (517L)"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

15 ACATTTTGCT GCCGGTCACT AGTGGTC

27

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:

- 20 (A) LENGTH: 27 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

- (A) DESCRIPTION: /desc = "end specific
adaptor (560L)"

25 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

ACATTTTGCT GCCGGTCACT AGTGGTA

27

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:

- 30 (A) LENGTH: 27 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

- 35 (A) DESCRIPTION: /desc = "end specific
adaptor (1567L)"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

ACATTTTGCT GCCGGTCACT AGTGATA

27

5 (2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:

- 10 (A) LENGTH: 27 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

- (A) DESCRIPTION: /desc = "end specific
adaptor (2684L) "

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

15 ACATTTTGCT GCCGGTCACT AGTAGTC

27

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:

- 20 (A) LENGTH: 27 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

- (A) DESCRIPTION: /desc = "end specific
adaptor (4459L) "

25 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

ACATTTTGCT GCCGGTCACT AGTGGGC

27

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:

- 30 (A) LENGTH: 27 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

- 35 (A) DESCRIPTION: /desc = "end specific
adaptor (4623L) "

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

ACATTTTGCT GCCGGTCACT AGTCAAG

27

5 (2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 27 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

10

(ii) MOLECULE TYPE: other nucleic acid

- (A) DESCRIPTION: /desc = "end specific
adaptor (6330L) "

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

15 ACATTTTGCT GCCGGTCACT AGTCAAA

27

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 27 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

20

(ii) MOLECULE TYPE: other nucleic acid

- (A) DESCRIPTION: /desc = "end specific
adaptor (18909L) "

25 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

ACATTTTGCT GCCGGTCACT AGTCGGC

27

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 18 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

30

(ii) MOLECULE TYPE: other nucleic acid

- (A) DESCRIPTION: /desc = "M13RevP reverse primer"

35 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

CAGGAAACAG CTATGACC

18

5 (2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:

- 10 (A) LENGTH: 27 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

- (A) DESCRIPTION: /desc = "end specific
adaptor (517R) "

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

15 GTCCTTTGTC GATACTGGCT AGTGAAG

27

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:

- 20 (A) LENGTH: 27 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

- (A) DESCRIPTION: /desc = "end specific
adaptor (1576R) "

25 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

GTCCTTTGTC GATACTGGCT AGTCAGT

27

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:

- 30 (A) LENGTH: 27 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

- 35 (A) DESCRIPTION: /desc = "end specific
adaptor (2684R) "

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

GTCCTTTGTC GATACTGGCT AGTCGGA

27

5 (2) INFORMATION FOR SEQ ID NO:14:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 27 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
10 (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "end specific
adaptor (4459R)"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

15 GTCCTTTGTC GATACTGGCT AGTGGAG

27

(2) INFORMATION FOR SEQ ID NO:15:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 27 base pairs
(B) TYPE: nucleic acid
20 (C) STRANDEDNESS: single
(D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "end specific
adaptor (4623R)"

25 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

GTCCTTTGTC GATACTGGCT AGTTCCT

27

(2) INFORMATION FOR SEQ ID NO:16:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 27 base pairs
(B) TYPE: nucleic acid
30 (C) STRANDEDNESS: single
(D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "end specific
35 adaptor (6330R)"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

GTCCTTTGTC GATACTGGCT AGTTGAC

27

5 (2) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:

- 10 (A) LENGTH: 27 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

- (A) DESCRIPTION: /desc = "end specific
adaptor (8848R) "

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

15 GTCCTTTGTC GATACTGGCT AGTTTAG

27

(2) INFORMATION FOR SEQ ID NO:18:

(i) SEQUENCE CHARACTERISTICS:

- 20 (A) LENGTH: 27 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

- (A) DESCRIPTION: /desc = "end specific
adaptor (18909R) "

25 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

GTCCTTTGTC GATACTGGCT AGTGGTG

27

(2) INFORMATION FOR SEQ ID NO:19:

(i) SEQUENCE CHARACTERISTICS:

- 30 (A) LENGTH: 27 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

- 35 (A) DESCRIPTION: /desc = "combinatorial adaptor
invading strand for forward primer"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

ACATTTTGCT GCCGGTCACT AGTNNNN

27

5 (2) INFORMATION FOR SEQ ID NO:20:

(i) SEQUENCE CHARACTERISTICS:

- 10 (A) LENGTH: 27 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

- (A) DESCRIPTION: /desc = "combinatorial adaptor
invading strand for reverse primer"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

15 GTCCTTTGTC GATACTGGCT AGTNNNN

27

(2) INFORMATION FOR SEQ ID NO:21:

(i) SEQUENCE CHARACTERISTICS:

- 20 (A) LENGTH: 18 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

- (A) DESCRIPTION: /desc = "lambda terminal primer
(right end)"

25 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

CGTAACCTGT CGGATCAC

18

(2) INFORMATION FOR SEQ ID NO:22:

(i) SEQUENCE CHARACTERISTICS:

- 30 (A) LENGTH: 18 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

- (A) DESCRIPTION: /desc = "lambda primer (left end)"

35 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

CGCGGGTTTT CGCTATTT

18

5 (2) INFORMATION FOR SEQ ID NO:23:

(i) SEQUENCE CHARACTERISTICS:

- 10 (A) LENGTH: 26 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(A) DESCRIPTION: /desc = "end specific adaptor"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

ACATTTTGCT GCCGGTCACT AGGACC

26

15 (2) INFORMATION FOR SEQ ID NO:24:

(i) SEQUENCE CHARACTERISTICS:

- 20 (A) LENGTH: 26 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(A) DESCRIPTION: /desc = "end specific adaptor"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:24:

ACATTTTGCT GCCGGTCACT AGCGAC

26

25 (2) INFORMATION FOR SEQ ID NO:25:

(i) SEQUENCE CHARACTERISTICS:

- 30 (A) LENGTH: 26 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(A) DESCRIPTION: /desc = "end specific adaptor"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:25:

ACATTTTGCT GCCGGTCACT AGCCGA

26

5 (2) INFORMATION FOR SEQ ID NO:26:

(i) SEQUENCE CHARACTERISTICS:

- 10 (A) LENGTH: 26 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

- (A) DESCRIPTION: /desc = "end specific adaptor"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:26:

ACATTTTGCT GCCGGTCACT AGGAGA

26

15 (2) INFORMATION FOR SEQ ID NO:27:

(i) SEQUENCE CHARACTERISTICS:

- 20 (A) LENGTH: 22 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

- (A) DESCRIPTION: /desc = "M13 reverse primer with
NlaIII adaptor"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:27:

25 CAGGAAACAG CTATGACCCA TG

22

(2) INFORMATION FOR SEQ ID NO:28:

(i) SEQUENCE CHARACTERISTICS:

- 30 (A) LENGTH: 18 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

- (A) DESCRIPTION: /desc = "adaptor strand"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:28:

35 GTCCTTTGTC GATACTGG

18

(2) INFORMATION FOR SEQ ID NO:29:

(i) SEQUENCE CHARACTERISTICS:

- 40 (A) LENGTH: 27 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

5 (ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "invading and primer strand
for 3'-overhang adaptor"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:29:
NNNNCTGCAT GACCGGCAGC AAAATGT 27

10 (2) INFORMATION FOR SEQ ID NO:30:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
15 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "oligonucleotide
complementary to M13 forward primer"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:30:
20 ACATTTTGCT GCCGGTCA 18

(2) INFORMATION FOR SEQ ID NO:31:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 27 base pairs
(B) TYPE: nucleic acid
25 (C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "oligonucleotide
30 complementary to M13 forward primer after
ligation to 3' overhang restriction
fragment end"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:31:
ACATTTTGCT GCCGGTCATG CAGNNNN 27

(2) INFORMATION FOR SEQ ID NO:32:

35 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 40 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

40 (ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "oligonucleotide
anchor primer for cDNA synthesis and PCR
primer in fragment amplification step"

5 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:32:

CAGGGTAGAC GACGCTACGC TTTTTTTTTT TTTTTTTTAT

40

5

CLAIMS

WE CLAIM:

1. A method for indexing polynucleotides, comprising the steps of:

(A) combining under base-pairing conditions:

10 (i) one or more distinguishable sets of indexing adaptors, each adaptor comprising at least one terminus having a single-stranded region characterized by, in non-overlapping order inward from the end: (a) an indexing sequence n bases long and (b) a sequence characteristic of
15 cleavage by a Class II restriction endonuclease, wherein n is an integer and wherein further each set of adaptors comprises one or more indexing sequences, and

(ii) one or more corresponding polynucleotides, each of which comprises at least one terminus characterized by,
20 in non-overlapping order inward from the end: (a) a region having a sequence characteristic of cleavage by a Class II restriction endonuclease and (b) a double-stranded region having on one strand a sequence that base-pairs with an indexing sequence of an adaptor and on the other strand a
25 sequence complementary thereto;

(B) base-pairing at least one adaptor terminus and at least one corresponding polynucleotide terminus to form at least one strand-displaced structure wherein the indexing sequence of the single-stranded region of the adaptor terminus
30 is base-paired with the sequence that base-pairs with an indexing sequence of the terminus of the corresponding polynucleotide, and the complementary sequence on the other strand of the polynucleotide terminus is displaced from base-pairing thereto;

35 (C) for each adaptor set, distinguishing the corresponding polynucleotides that form strand-displaced structures, thereby indexing the polynucleotides by their base-pairing to the distinguishable sets of adaptors.

2. A method according to claim 1, wherein n is 1 to 4.

5 3. A method according to claim 2 wherein n is 2, 3 or 4.

4. A method according to claim 1, wherein the indexing adaptors in all the sets of adaptors together comprise all possible sequences of A, T, G and C n bases long.

10 5. A method according to claim 1, further comprising the step of ligating, in a strand-displaced structure, the end of the adaptor strand comprising the indexing sequence to the end of the abutting strand of the double-stranded region of the corresponding polynucleotide.

15 6. A method according to claim 1, wherein one or more adaptors further comprise a sequence for amplification.

7. A method according to claim 1, wherein strand-displaced structures are formed at both ends of at least one corresponding polynucleotide.

20 8. A method according to claim 7, wherein the adaptor at each terminus of at least one corresponding polynucleotide further comprises at least one sequence for amplification by PCR.

25 9. A method according to claim 8, further comprising the step of ligating, in the strand-displaced structure of each terminus of at least one polynucleotide, the end of the adaptor strand comprising the indexing sequence to the end of the abutting strand of the double-stranded region of the corresponding polynucleotide.

30 10. A method according to claim 9, further comprising the step of amplifying by PCR corresponding polynucleotides having adaptor strands ligated to each terminus, using the adaptor sequences for amplification.

5 11. A method according to claim 6, further comprising the
step of amplifying at least one polynucleotide using primers
defined by the sequence for amplification.

 12. A method according to claim 1, wherein the sequence
characteristic of cleavage by a Class II restriction
10 endonuclease has a 3'-terminated single-stranded region.

 13. A method according to claim 1, wherein the sequence
characteristic of cleavage by a Class II restriction
endonuclease has a 5'-terminated single-stranded region.

 14. A method according to claim 1, wherein the sequence
15 characteristic of cleavage by a Class II restriction
endonuclease has a blunt end.

5 15. A method for characterizing a population of polynucleotides, comprising the steps of:

(A) combining under base-pairing conditions:

10 (i) one or more distinguishable sets of indexing adaptors, each adaptor comprising at least one terminus having a single-stranded region characterized by, in non-overlapping order inward from the end: (a) an indexing sequence n bases long and (b) a sequence characteristic of cleavage by a Class II restriction endonuclease, wherein n is an integer and wherein further each set of adaptors
15 comprises one or more indexing sequences, and

 (ii) a population of polynucleotides that includes one or more corresponding polynucleotides, each of which comprises at least one terminus characterized by, in non-overlapping order inward from the end: (a) a region having
20 a sequence characteristic of cleavage by a Class II restriction endonuclease and (b) a double-stranded region having on one strand a sequence that base-pairs with an indexing sequence of an adaptor and on the other strand a sequence complementary thereto;

25 (B) base-pairing at least one adaptor terminus to at least one corresponding polynucleotide terminus to form at least one strand-displaced structure wherein the indexing sequence of the single-stranded region of the adaptor terminus is base-paired with the sequence that base-pairs with an
30 indexing sequence of the terminus of the corresponding polynucleotide, and the complementary sequence on the other strand of the polynucleotide terminus is displaced from base-pairing thereto;

35 (C) for each adaptor set, determining one or more corresponding polynucleotides that form strand-displaced structures, or the absence thereof;

(D) characterizing the population of polynucleotides by the corresponding polynucleotides that form strand-displaced structures, or the absence thereof.

5 16. A method according to claim 15, wherein n is 1 to 5.

 17. A method according to claim 15, wherein the population is a population of cDNAs or other polynucleotides representative of mRNAs.

10 18. A method according to claim 17, wherein the characterization is indicative of gene expression in a sample from which the population was derived.

 19. A method according to claim 15, wherein the population is a population of genomic DNAs or polynucleotides representative of genomic DNAs.

15 20. A method according to claim 19, wherein the characterization comprises characterizing mutations or the absence thereof in: (a) the sequence characteristic of cleavage by a Class II restriction endonuclease; (b) the indexing sequence or both (a) and (b) of one or more corresponding
20 polynucleotides in the population.

 21. A method according to claim 20, wherein the absence or presence of a mutation thus characterized in one or more corresponding polynucleotides is diagnostic of a potential to develop one or more diseases, or of one or more diseases.

25 22. A method according to claim 15, further comprising the step of ligating, in a strand-displaced structure, the end of the adaptor-strand comprising the indexing sequence to the end of the abutting strand of the double-stranded region of the corresponding polynucleotide.

30 23. A method according to claim 15, wherein one or more adaptors further comprise a sequence for amplification.

5 24. A method according to claim 15, wherein strand-displaced structures are formed at both ends of at least one corresponding polynucleotide.

10 25. A method according to claim 24, wherein the adaptor at each terminus of at least one corresponding polynucleotide further comprises at least one sequence for amplification by PCR.

15 26. A method according to claim 25, further comprising the step of ligating, in the strand-displaced structure of each terminus of at least one polynucleotide, the end of the adaptor strand comprising the indexing sequence to the end of the abutting strand of the double-stranded region of the corresponding polynucleotide.

20 27. A method according to claim 26, further comprising the step of amplifying by PCR corresponding polynucleotides having adaptor strands ligated to each terminus, using the adaptor sequences for amplification either as a primer or as a primer binding site.

25 28. A method according to claim 15, further comprising, for at least one adaptor set, resolving from one another at least two corresponding polynucleotides that form strand-displaced structures.

5 29. A method according to claim 28, wherein the
population is a population of cDNAs or other polynucleotides
representative of mRNAs in a sample, the size and the quantity
of the separated corresponding polynucleotides is determined,
each separated corresponding polynucleotide is identified by
10 the indexing sequence and the Class II restriction endonuclease
characteristic sequence of the adaptor set with which it formed
a strand-displaced structure and by its size, and the
quantities of the thus identified corresponding polynucleotides
for at least two adaptor sets provides a profile of gene
15 expression in the source from which the cDNA was derived.

30. A method according to claim 28, wherein corresponding
polynucleotides for at least one set of adaptors are resolved
by size by electrophoresis.

20 31. A method according to claim 29, wherein the sequences
characteristic of a Class II restriction endonuclease and the
indexing sequences of the adaptor sets together subdivide the
cDNAs or other polynucleotides representative of the mRNAs into
sets of corresponding polynucleotides in each of which
corresponding polynucleotides can be individually
25 differentiated by electrophoresis.

32. A method for comparing mRNA in two or more samples,
comprising the steps of claim 29 to generate a profile of gene
expression of a first sample and, independently, a profile of
gene expression in a second sample and comparing the profiles
30 of the first and second samples.

33. A set of adaptors, each adaptor comprising at least
one terminus having a single-stranded region characterized by,
in non-overlapping order inward from the end: (a) an indexing
sequence n bases long and (b) a sequence characteristic of
35 cleavage by a Class II restriction endonuclease, wherein n is
an integer, the set of adaptors comprising adaptors with at
least two of the possible indexing sequences n bases long.

5 34. A set of adaptors according to claim 33, wherein n is
1, 2, 3, 4 or 5.

35. A set of adaptors according to claim 34, wherein n is
2, 3 or 4.

10 36. A set of adaptors according to claim 33, wherein the
bases are A, C, G and T.

37. A set of adaptors according to claim 33, wherein the
bases are selected from the group consisting of A, C, G, T and
modified bases.

15 38. A set of adaptors according to claim 33, comprising
adaptors with all possible indexing sequences of A, C, G or T n
bases long.

39. A set of adaptors according to claim 38, wherein n is
1, 2, 3, 4 or 5.

20 40. A set of adaptors according to claim 39, wherein n is
2, 3 or 4.

41. A set of adaptors according to claim 33, comprising
adaptors with all possible indexing sequences n bases long, the
bases being one or more of the group consisting of A, C, G, T
and modified bases.

5 42. A method for amplifying a polynucleotide, comprising the steps of:

(A) combining under base-pairing conditions:

10 (1) an adaptor comprising: (a) at least one terminus having a single-stranded region characterized by, in non-overlapping order inward from the end, (i) a sequence n bases long for base-pairing to one or more polynucleotides, where n is an integer and (ii) a sequence characteristic of cleavage by a Class II restriction endonuclease, and (b) a region comprising a sequence for
15 amplifying a polynucleotide, and

 (2) a polynucleotide comprising at least one terminus characterized by, in non-overlapping order inward from the end, (a) a region having a sequence characteristic of cleavage by a Class II restriction
20 endonuclease and (b) a double-stranded region having on one strand a sequence that base-pairs with the single-stranded adaptor sequence for base-pairing with a polynucleotide, and on the other strand a sequence complementary thereto;

25 (B) base-pairing the single-stranded adaptor sequence for base-pairing to one or more polynucleotides with the polynucleotide sequence that base-pairs therewith and displacing the complementary sequence on the other strand of the polynucleotide terminus;

30 (C) ligating the end of the adaptor strand comprising the indexing sequence to the end of the abutting strand of the double-stranded region of the polynucleotide; and

 (D) amplifying the polynucleotide using the adaptor sequence for amplifying a polynucleotide.

35 43. A method according to claim 42, wherein the sequence for amplifying a polynucleotide is selected from the group consisting of a primer for elongating a template by a DNA polymerase and a sequence complementary to a primer for elongating a template by a DNA polymerase.

5 44. A method according to 43, wherein adaptors are ligated at both ends of the polynucleotide and exponential amplification of the polynucleotide is carried out by PCR using primers defined by the sequences of the adaptors for amplifying a polynucleotide.

10 45. A method according to claim 42, wherein more than two adaptors each having a different sequence for base-pairing with a polynucleotide are used to base pair specifically to different polynucleotides in a population of polynucleotides.

15 46. A method according to claim 42, wherein an adaptor is ligated to each end of the polynucleotides and exponential amplification of the polynucleotides is carried out by PCR using primers defined by the sequences for amplification of the adaptors.

20 47. A method according to claim 46, wherein the sequences of the adaptors for base-pairing to one or more polynucleotides together comprise all sequences of A, C, G, and T n bases long.

5 48. A method for isolating a polynucleotide, comprising the steps of:

(A) combining under base-pairing conditions:

10 (1) an adaptor comprising: (a) at least one terminus having a single-stranded region characterized by, in non-overlapping order inward from the end, (i) a sequence n bases long for base-pairing to one or more polynucleotides, where n is an integer and (ii) a sequence characteristic of cleavage by a Class II restriction endonuclease, and (b) a region comprising a sequence for
15 amplifying a polynucleotide, and

(2) a polynucleotide comprising at least one terminus characterized by, in non-overlapping order inward from the end, (a) a region having a sequence characteristic of cleavage by a Class II restriction
20 endonuclease and (b) a double-stranded region having on one strand a sequence that base-pairs with the single-stranded adaptor sequence for base-pairing with a polynucleotide, and on the other strand a sequence complementary thereto;

25 (B) base-pairing the single-stranded adaptor sequence for base-pairing to a polynucleotide with the polynucleotide sequence that base-pairs therewith and displacing the complementary sequence on the other strand of the polynucleotide terminus;

30 (C) ligating the end of the adaptor strand comprising the indexing sequence to the end of the abutting strand of the double-stranded region of the polynucleotide;

(D) amplifying the polynucleotide using the adaptor sequence for amplifying a polynucleotide; and

35 (E) isolating the amplified polynucleotide.

40 49. A method according to claim 48, wherein the sequence for amplifying a polynucleotide is selected from the group consisting of a primer for elongating a template by a DNA polymerase and a sequence complementary to a primer for elongating a template by a DNA polymerase.

5 50. A method according to 49, wherein adaptors are ligated at both ends of the polynucleotide and exponential amplification of the polynucleotide is carried out by PCR using primers defined by the sequences of the adaptors for amplifying a polynucleotide.

10 51. A method according to claim 48, wherein two or more adaptors each having a different sequence for base-pairing with a polynucleotide are used to base pair specifically to two or more different polynucleotides in a population of polynucleotides.

15 52. A method according to claim 51, wherein an adaptor is ligated to each end of the polynucleotides and exponential amplification of the polynucleotides is carried out by PCR using primers defined by the sequences of the adaptors for amplifying a polynucleotide.

20 53. A method according to claim 48, wherein the amplified polynucleotide is resolved from other polynucleotides by gel electrophoresis and then eluted from the gel.

 54. A method according to claim 48, further comprising the step of cloning the amplified polynucleotide.

5 55. One or more kits together comprising a plurality of
adaptors, wherein:

 (A) each adaptor comprises at least one terminus having a
single-stranded region characterized by, in non-overlapping
order inward from the end, (a) an indexing sequence n bases
10 long wherein n is an integer, and (b) a sequence characteristic
of cleavage by a Class II restriction endonuclease, and

 (B) the plurality of adaptors comprises for each given
sequence characteristic of cleavage by a Class II restriction
endonuclease at least one adaptor having at least one specific
15 indexing sequence.

 56. A kit or kits according to claim 55, wherein the
sequence characteristic of cleavage by a Class II restriction
endonuclease is selected from the group consisting of a
sequence having a 5' overhang and a sequence having a 3'
20 overhang.

 57. A kit or kits according to claim 56, wherein the
Class II restriction endonuclease is selected from the group
consisting of BclI, NotI, DpnII, BamHI, HindIII, AvrII, ApaLI,
KpnI, SphI, NsiI, and SacI.

25 58. A kit or kits according to claim 56, wherein n is 1,
2 or 3.

 59. A kit or kits according to claim 56, wherein the
plurality of adaptors comprises a set of adaptors with indexing
sequences that base-pair with each sequence n bases long of A,
30 C, G, and T, where n is an integer.

5 60. A kit or kits according to claim 59, wherein the
base-pairing specificity of each base of the adaptor indexing
sequences is selected from the group consisting of A, C, G, T,
Py, Pu and N, wherein Py denotes base pairing to A and G, Pu
denotes base-pairing to C and T and N denotes base-pairing to
10 A, C, G and T.

 61. A kit or kits according to claim 60, wherein the
bases of the indexing sequences are selected from the group
consisting of A, C, T, G and X, where X is a nucleoside other
than A, C, T or G that can form specific base-pairs with A, C,
15 G or T in DNA.

 62. A kit or kits according to 61, wherein X contains the
modified base 3'-nitropyrrole or the modified base 5'-
nitroindole.

 63. A kit or kits according to claim 61, wherein the
20 plurality of adaptors comprises a set of adaptors having each
indexing sequence n bases long of A, C, G and T, where n is an
integer.

5 64. A method for indexing polynucleotides, comprising the steps of:

(A) combining under base-pairing conditions:

10 (i) one or more distinguishable sets of indexing adaptors, each adaptor comprising a sequence for amplification and at least one terminus having a single-stranded region characterized by, in non-overlapping order inward from the end: (a) an indexing sequence n bases long and (b) a sequence characteristic of cleavage by a Class II restriction endonuclease, wherein n is an integer and
15 wherein further each set of adaptors comprises one or more indexing sequences, and

20 (ii) one or more corresponding polynucleotides, each of which comprises at least one terminus characterized by, in non-overlapping order inward from the end: (a) a region having a sequence characteristic of cleavage by a Class II restriction endonuclease and (b) a double-stranded region having on one strand a sequence that base-pairs with an indexing sequence of an adaptor and on the other strand a sequence complementary thereto;

25 (B) base-pairing at least one adaptor terminus and at least one corresponding polynucleotide terminus to form at least one strand-displaced structure wherein the indexing sequence of the single-stranded region of the adaptor terminus is base-paired with the sequence that base-pairs with an
30 indexing sequence of the terminus of the corresponding polynucleotide, and the complementary sequence on the other strand of the polynucleotide terminus is displaced from base-pairing thereto;

35 (C) for each adaptor set, amplifying the corresponding polynucleotides that form strand-displaced structures using the sequence for amplification; and

(D) distinguishing the amplified corresponding polynucleotides thereby indexing the polynucleotides by their base-pairing to the distinguishable sets of adaptors.

40 65. A method according to claim 64, wherein n is 1 to 4.

5 66. A method according to claim 65 wherein n is 2, 3 or
4.

67. A method according to claim 64, wherein the indexing
adaptors in all the sets of adaptors together comprise all
possible sequences of A, T, G and C n bases long.

10 68. A method according to claim 64, wherein the sequence
for amplification is selected from a group consisting of a PCR
primer, a T3 promoter, a T7 promoter, an SP6 promoter, and a
sequence complementary to any of the same.

15 69. A method according to claim 64, wherein the
amplification is an exponential amplification.

20 70. A method according to claim 64, further comprising
the step of ligating, in a strand-displaced structure, the end
of the adaptor strand comprising the indexing sequence to the
end of the abutting strand of the double-stranded region of the
corresponding polynucleotide.

71. A method according to claim 70, wherein strand-
displaced structures are formed at both ends of at least one
corresponding polynucleotide.

25 72. A method according to claim 71, wherein the sequence
for amplification is a PCR primer.

73. A method according to claim 64, wherein the sequence
characteristic of cleavage by a Class II restriction
endonuclease has a 3'-terminated single-stranded region.

30 74. A method according to claim 64, wherein the sequence
characteristic of cleavage by a Class II restriction
endonuclease has a 5'-terminated single-stranded region.

5 75. A method according to claim 64, wherein the sequence characteristic of cleavage by a Class II restriction endonuclease has a blunt end.

76. A method for characterizing a population of polynucleotides, comprising the steps of:

10 (A) combining under base-pairing conditions:

 (i) one or more distinguishable sets of indexing adaptors, each adaptor comprising a sequence for amplification and at least one terminus having a single-stranded region characterized by, in non-overlapping order inward from the end: (a) an indexing sequence n bases long and (b) a sequence characteristic of cleavage by a Class II restriction endonuclease, wherein n is an integer and wherein further each set of adaptors comprises one or more indexing sequences, and

20 (ii) a population of polynucleotides that includes one or more corresponding polynucleotides, each of which comprises at least one terminus characterized by, in non-overlapping order inward from the end: (a) a region having a sequence characteristic of cleavage by a Class II restriction endonuclease and (b) a double-stranded region having on one strand a sequence that base-pairs with an indexing sequence of an adaptor and on the other strand a sequence complementary thereto;

25 (B) base-pairing at least one adaptor terminus to at least one corresponding polynucleotide terminus to form at least one strand-displaced structure wherein the indexing sequence of the single-stranded region of the adaptor terminus is base-paired with the sequence that base-pairs with an indexing sequence of the terminus of the corresponding polynucleotide, and the complementary sequence on the other strand of the polynucleotide terminus is displaced from base-pairing thereto;

35 (C) for each adaptor set, amplifying corresponding polynucleotides that form strand-displaced structures using the sequence for amplification;

40

5 (D) determining one or more amplified corresponding polynucleotides or the absence thereof; and

(E) characterizing the population of polynucleotides by the amplified corresponding polynucleotides that form strand-displaced structures using the sequence for amplification, or
10 the absence thereof.

77. A method according to claim 76, wherein n is 1 to 5.

78. A method according to claim 76, wherein the population is a population of cDNAs or other polynucleotides representative of mRNAs.

15 79. A method according to claim 78, wherein the characterization is indicative of gene expression in a sample from which the population was derived.

80. A method according to claim 76, wherein the population is a population of genomic DNAs or polynucleotides representative of genomic DNAs.
20

81. A method according to claim 80, wherein the characterization comprises characterizing mutations or the absence thereof in: (a) the sequence characteristic of cleavage by a Class II restriction endonuclease; (b) the indexing
25 sequence or both (a) and (b) of one or more corresponding polynucleotides in the population.

82. A method according to claim 81, wherein the absence or presence of a mutation thus characterized in one or more corresponding polynucleotides is diagnostic of a potential to
30 develop one or more diseases, or of one or more diseases.

83. A method according to claim 76, further comprising the step of ligating, in a strand-displaced structure, the end of the adaptor-strand comprising the indexing sequence to the end of the abutting strand of the double-stranded region of the

5 corresponding polynucleotide.

84. A method according to claim 83, wherein strand-displaced structures are formed at both ends of at least one corresponding polynucleotide.

10 85. A method according to claim 84, wherein the sequence for amplification is a PCR primer.

15 86. A method according to claim 85, further comprising the step of amplifying by PCR corresponding polynucleotides having adaptor strands ligated to each terminus, using the sequences for amplification either as a primer or as a primer binding site.

87. A method according to claim 76, further comprising, for at least one adaptor set, resolving from one another at least two corresponding polynucleotides that form strand-displaced structures.

20 88. A method according to claim 87, wherein the population is a population of cDNAs or other polynucleotides representative of mRNAs in a sample, the size and the quantity of the separated corresponding polynucleotides is determined, each separated corresponding polynucleotide is identified by
25 the indexing sequence and the Class II restriction endonuclease characteristic sequence of the adaptor set with which it formed a strand-displaced structure and by its size, and the quantities of the thus identified corresponding polynucleotides for at least two adaptor sets provides a profile of gene
30 expression in the source from which the cDNA was derived.

89. A method according to claim 88, wherein corresponding polynucleotides for at least one set of adaptors are resolved by size by electrophoresis.

5 90. A method according to claim 88, wherein the sequences
characteristic of a Class II restriction endonuclease and the
indexing sequences of the adaptor sets together subdivide the
cDNAs or other polynucleotides representative of the mRNAs into
10 sets of corresponding polynucleotides in each of which
corresponding polynucleotides can be individually
differentiated by electrophoresis.

 91. A method for comparing mRNA in two or more samples,
comprising the steps of claim 88 to generate a profile of gene
expression of a first sample and, independently, a profile of
15 gene expression in a second sample and comparing the profiles
of the first and second samples.

 92. A method according to claim 76, wherein the sequence
for amplification is selected from a group consisting of a PCR
primer, a T3 promoter, a T7 promoter, an SP6 promoter, and a
20 sequence complementary to same.

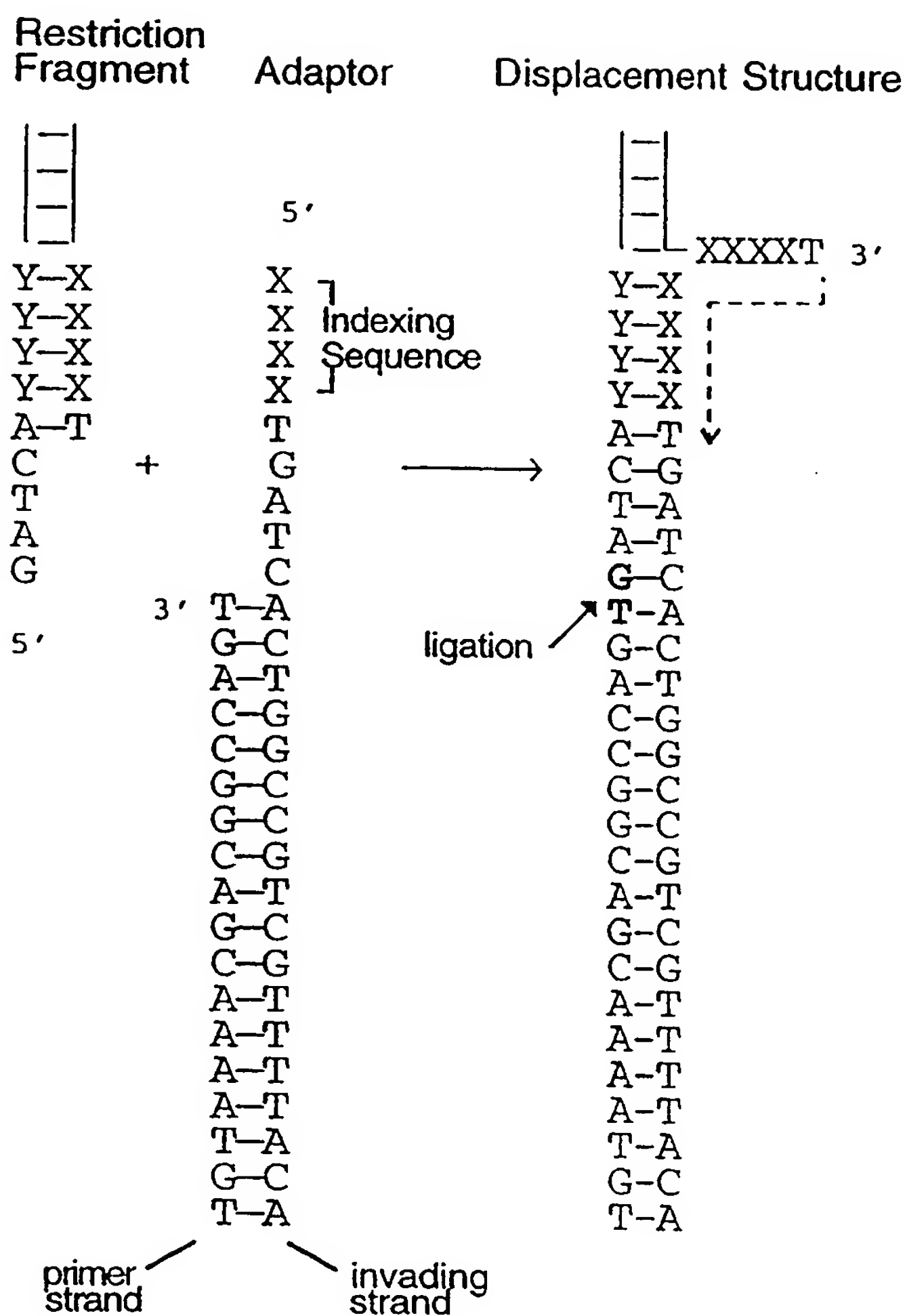


FIG 1

2/5

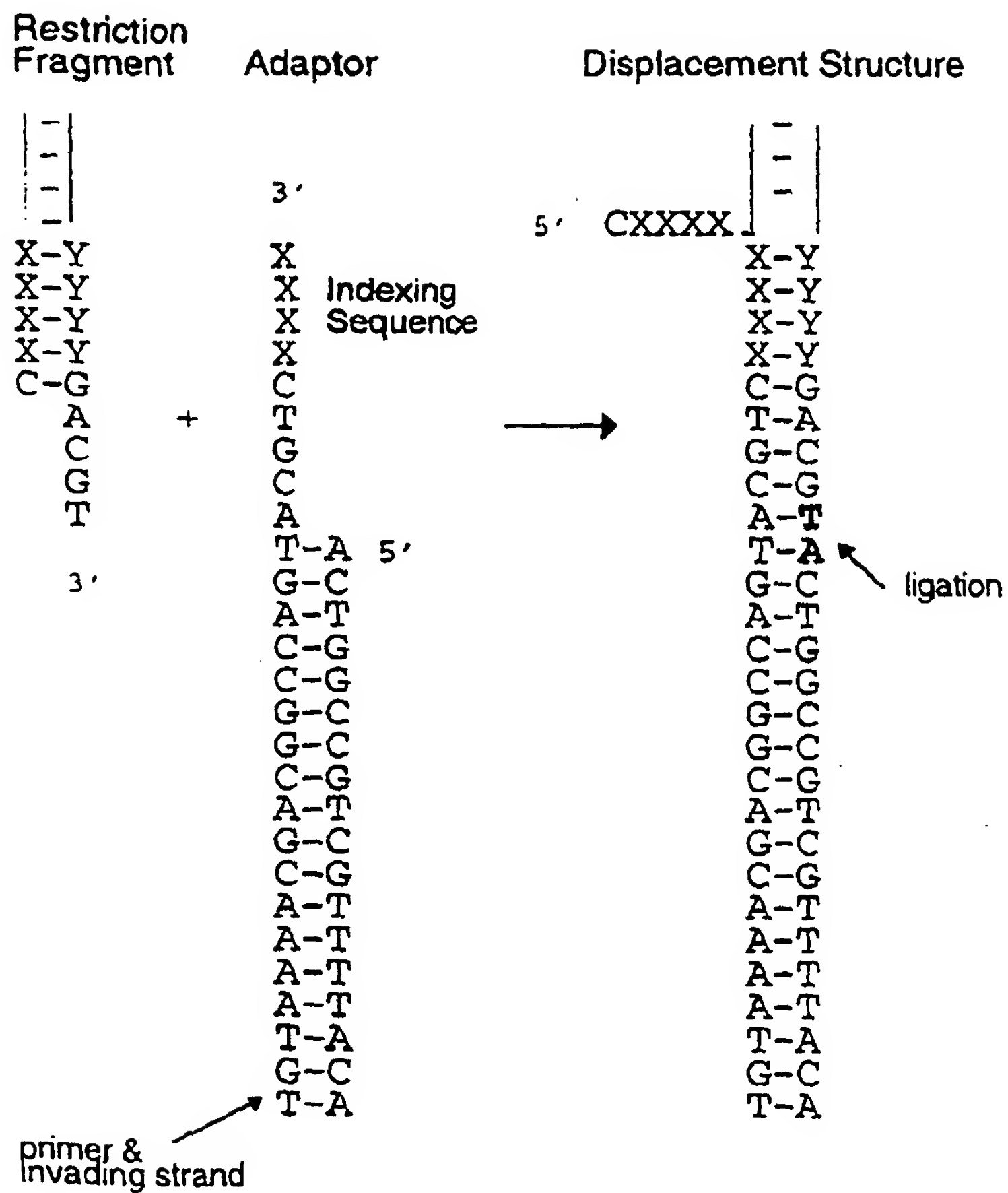


FIG 2

RECTIFIED SHEET (RULE 91)
ISA/EP

3/5

A. *END-SPECIFIC ADAPTORS*LEFT w/forward primer

-21M13	5'	tgtaaaacgacggccagt
517L	3'	ACATTTTGCTGCCGGTCACTAGTGGTC
560L		ACATTTTGCTGCCGGTCACTAGTGGTA
1567L		ACATTTTGCTGCCGGTCACTAGTGATA
2684L		ACATTTTGCTGCCGGTCACTAGTAGTC
4459L		ACATTTTGCTGCCGGTCACTAGTGGGC
4623L		ACATTTTGCTGCCGGTCACTAGTCAAG
6330L		ACATTTTGCTGCCGGTCACTAGTCAA
18909L		ACATTTTGCTGCCGGTCACTAGTCGGC

RIGHT w/reverse primer

M13RevP	5'	caggaaacagctatgacc
517R	3'	GTCCTTTGTCGATACTGGCTAGTGAAG
1576R		GTCCTTTGTCGATACTGGCTAGTCAGT
2684R		GTCCTTTGTCGATACTGGCTAGTCGGA
4459R		GTCCTTTGTCGATACTGGCTAGTGGAG
4623R		GTCCTTTGTCGATACTGGCTAGTTCCT
6330R		GTCCTTTGTCGATACTGGCTAGTTGAC
8848R		GTCCTTTGTCGATACTGGCTAGTTTAG
18909R		GTCCTTTGTCGATACTGGCTAGTGGTG

B. *COMBINATORIAL ADAPTORS w/forward or reverse primer*

Combo-FP	5'	tgtaaaacgacggccagt
	3'	ACATTTTGCTGCCGGTCACTAGTNNNN
Combo-RP	5'	caggaaacagctatgacc
	3'	GTCCTTTGTCGATACTGGCTAGTNNNN

FIG 3

4/5

DpnII adaptors w/forward primer

```
5'      tgtaaaacgacggccagt
3'      ACATTTTGCTGCCGGTCACTAGGACC
        ACATTTTGCTGCCGGTCACTAGCGAC
        ACATTTTGCTGCCGGTCACTAGCCGA
        ACATTTTGCTGCCGGTCACTAGGAGA
```

NlaIII adaptor w/reverse primer

```
5'      CAGGAAACAGCTATGACCCATG
3'      GTCCTTTGTCGATACTGG
```

FIG 4

5/5

Sau3AI: 5' ∇ GATC
CTAG_A

tgtaaaacgacggccagt + GATCTTTT----- IL-2
ACATTTTGCTGCCGGTCACTAGAAAA AAAA-----

↓ T4 DNA Ligase

M13 forward →
tgtaaaacgacggccagtGATCTTTT-----//-----
ACATTTTGCTGCCGGTCACTAGAAAA┐-----//-----
A
A
A
A 3' ← N₁N₂(dt)₁₈ heel (AT)

200 = 26 + 142 (-8) + 40

Sau3AI indexing adaptors:

tgtaaaacgacggccagt	
ACATTTTGCTGCCGGTCACTAG GACC	IS-1
CGAC	IS-2
CCGA	IS-3
GAGA	IS-4
GA	IS-5
AGTC	IS-6
TATA	IS-7
ATAT	IS-8
AAAA	IS-9 (IL-2)

FIG 5

1/5

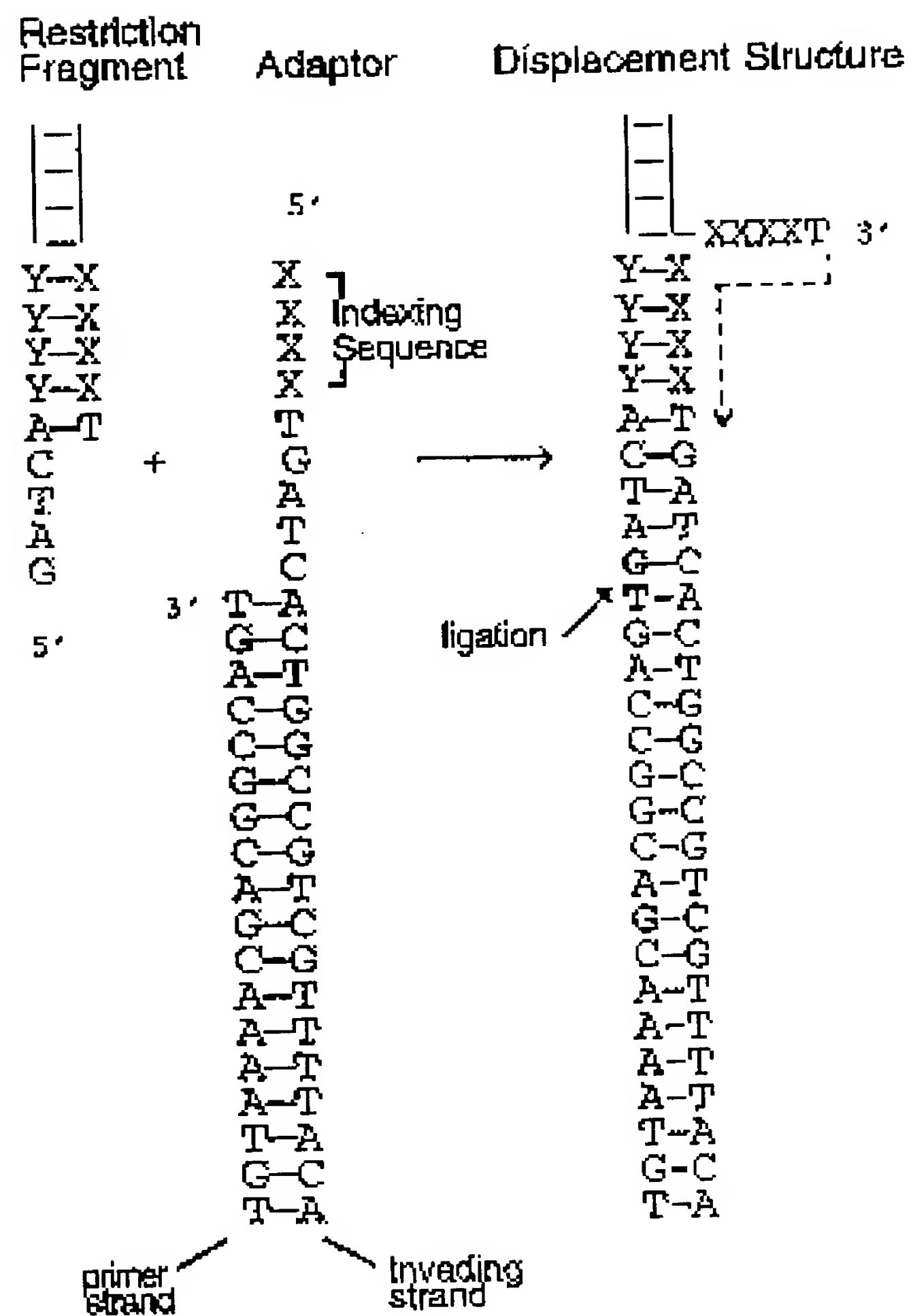


FIG 1

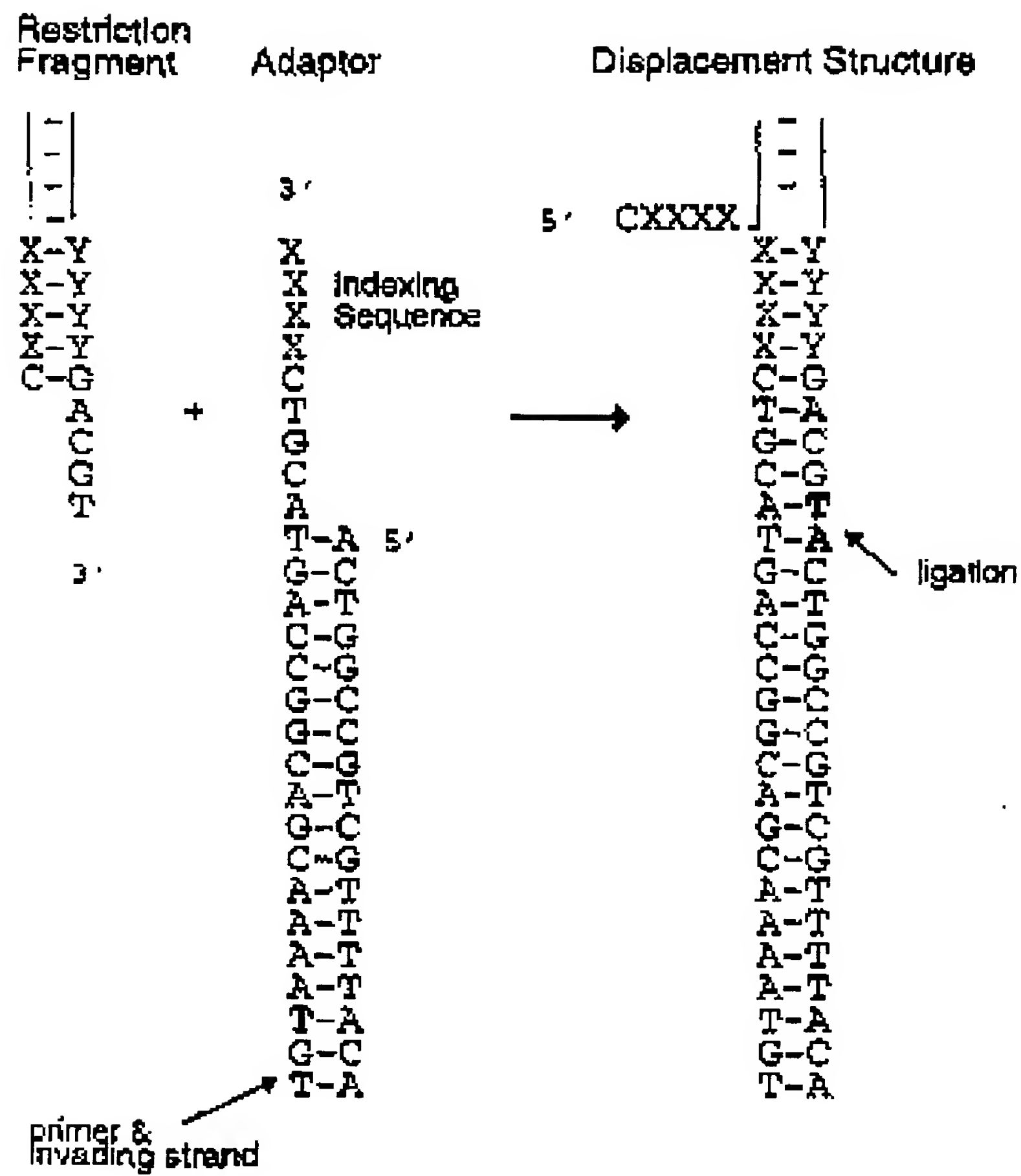


FIG 2

RECTIFIED SHEET (RULE 91)
ISA/EP

3/5

A. END-SPECIFIC ADAPTORSLEFT w/forward primer

-21M13	5'	tgtaaaacgacggccagt
517L	3'	ACATTTTGCTGCCGGTCACTAGTGGTC
560L		ACATTTTGCTGCCGGTCACTAGTGGTA
1567L		ACATTTTGCTGCCGGTCACTAGTGATA
2684L		ACATTTTGCTGCCGGTCACTAGTAGTC
4459L		ACATTTTGCTGCCGGTCACTAGTGGGC
4623L		ACATTTTGCTGCCGGTCACTAGTCAAG
6330L		ACATTTTGCTGCCGGTCACTAGTCAAA
18909L		ACATTTTGCTGCCGGTCACTAGTCGGC

RIGHT w/reverse primer

M13RevP	5'	caggaaacagctatgacc
517R	3'	GTCCTTTGTCGATACTGGCTAGTGAAG
1576R		GTCCTTTGTCGATACTGGCTAGTCAGT
2684R		GTCCTTTGTCGATACTGGCTAGTCGGA
4459R		GTCCTTTGTCGATACTGGCTAGTGGAG
4623R		GTCCTTTGTCGATACTGGCTAGTTCCT
6330R		GTCCTTTGTCGATACTGGCTAGTTGAC
8848R		GTCCTTTGTCGATACTGGCTAGTTTAG
18909R		GTCCTTTGTCGATACTGGCTAGTGGTG

B. COMBINATORIAL ADAPTORS w/forward or reverse primer

Combo-FP	5'	tgtaaaacgacggccagt
	3'	ACATTTTGCTGCCGGTCACTAGTNNNN
Combo-RP	5'	caggaaacagctatgacc
	3'	GTCCTTTGTCGATACTGGCTAGTNNNN

FIG 3

4/5

DpnII adaptors w/forward primer

```
5'      tgtaaaacgacggccagt
3'      ACATTTTGCTGCCGGTCACTAGGACC
        ACATTTTGCTGCCGGTCACTAGCGAC
        ACATTTTGCTGCCGGTCACTAGCCGA
        ACATTTTGCTGCCGGTCACTAGGAGA
```

NlaIII adaptor w/reverse primer

```
5'      CAGGAAACAGCTATGACCCATG
3'      GTCCTTTGTCGATACTGG
```

FIG 4

5/5

Sau3AI: 5' ∇ GATC
CTAG_A

tgtaaaacgacggccagt +
ACATTTTGCTGCCGGTCACTAGAAAA

GATCTTTT----- IL-2
AAAA-----

↓ T4 DNA Ligase

M13 forward →

tgtaaaacgacggccagtGATCTTTT-----//-----
ACATTTTGCTGCCGGTCACTAGAAAA-----//-----

A
A
A
A 3'

← N₃N₂(dt)₁₈ heol
(AT)

200 = 26 + 142 (-8) + 40

Sau3AI indexing adaptors:

tgtaaaacgacggccagt	
ACATTTTGCTGCCGGTCACTAGGACC	IS-1
CGAC	IS-2
CCGA	IS-3
GAGA	IS-4
GACT	IS-5
AGTC	IS-6
TATA	IS-7
ATAT	IS-8
AAAA	IS-9 (IL-2)

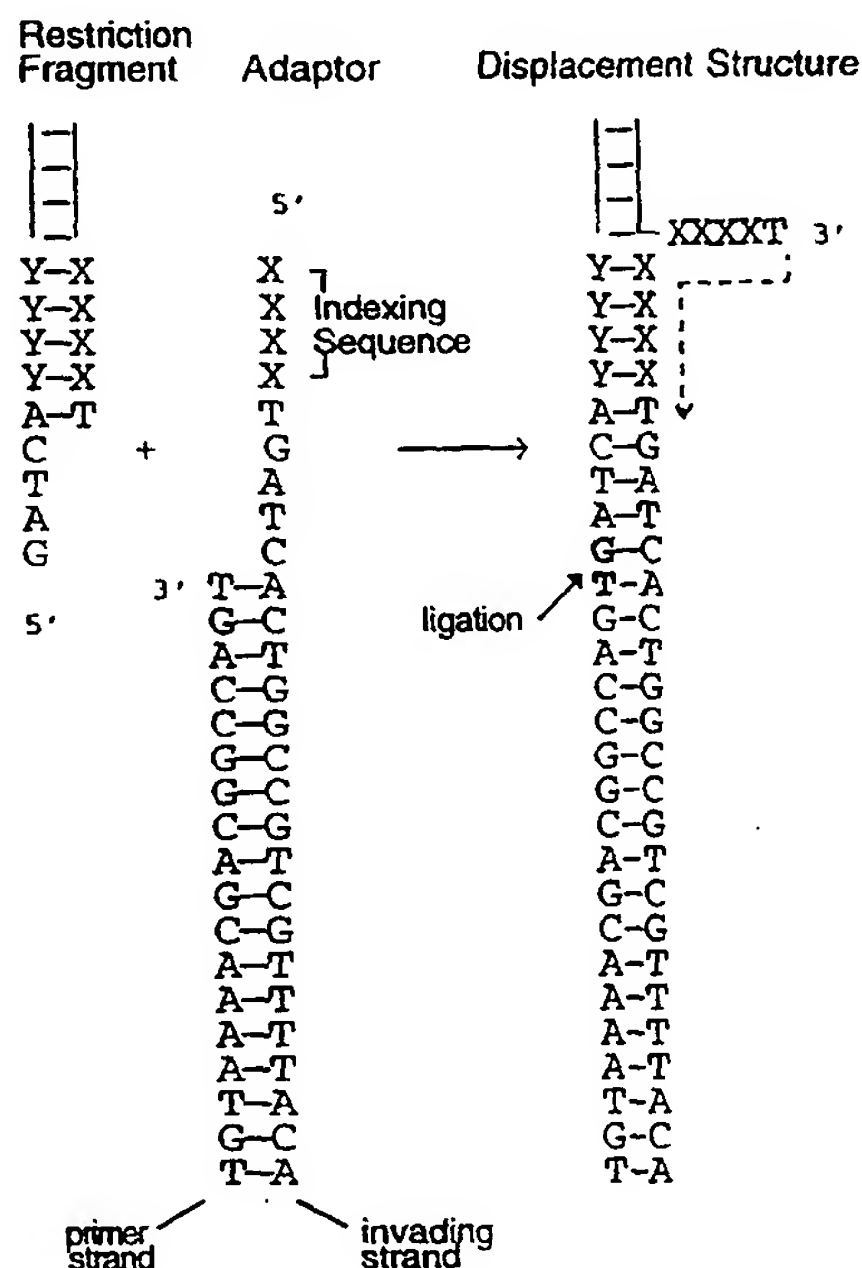
FIG 5



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification : C12Q 1/68	A3	(11) International Publication Number: WO 98/40518
		(43) International Publication Date: 17 September 1998 (17.09.98)
(21) International Application Number: PCT/US98/04819 (22) International Filing Date: 11 March 1998 (11.03.98) (30) Priority Data: 08/815,448 11 March 1997 (11.03.97) US (71) Applicant (<i>for all designated States except US</i>): WISCONSIN ALUMNI RESEARCH FOUNDATION [US/US]; 614 North Walnut Street, P.O. Box 7365, Madison, WI 53707-7365 (US). (72) Inventors; and (75) Inventors/Applicants (<i>for US only</i>): GUILFOYLE, Richard, A. [US/US]; 19912 Gateshead Circle, Germantown, MD 20876 (US). GUO, Zhen [CN/US]; Apartment 1044, 14611 N.E. 39th Street, Bellevue, WA 98007 (US). (74) Agent: BERSON, Bennett, J.; Quarles & Brady, P.O. Box 2113, Madison, WI 53701-2113 (US).		(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, GM, GW, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG). Published <i>With international search report.</i> (88) Date of publication of the international search report: 17 December 1998 (17.12.98)

(54) Title: NUCLEIC ACID INDEXING



(57) Abstract

A restriction site indexing method for selectively amplifying any fragment generated by a Class II restriction enzyme includes adaptors specific to fragment ends containing adaptor indexing sequences complementary to fragment indexing sequences near the termini of fragments generated by Class II enzyme cleavage. A method for combinatorial indexing facilitates amplification of restriction fragments whose sequence is not known. Profiling methods and other methods for characterizing polynucleotides are presented.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Latvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav Republic of Macedonia	TM	Turkmenistan
BF	Burkina Faso	GR	Greece	ML	Mali	TR	Turkey
BG	Bulgaria	HU	Hungary	MN	Mongolia	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MR	Mauritania	UA	Ukraine
BR	Brazil	IL	Israel	MW	Malawi	UG	Uganda
BY	Belarus	IS	Iceland	MX	Mexico	US	United States of America
CA	Canada	IT	Italy	NE	Niger	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NL	Netherlands	VN	Viet Nam
CG	Congo	KE	Kenya	NO	Norway	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NZ	New Zealand	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's Republic of Korea	PL	Poland		
CM	Cameroon	KR	Republic of Korea	PT	Portugal		
CN	China	KZ	Kazakstan	RO	Romania		
CU	Cuba	LC	Saint Lucia	RU	Russian Federation		
CZ	Czech Republic	LI	Liechtenstein	SD	Sudan		
DE	Germany	LK	Sri Lanka	SE	Sweden		
DK	Denmark	LR	Liberia	SG	Singapore		
EE	Estonia						

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 98/04819

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12Q1/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WONG D M ET AL: "BRANCH CAPTURE REACTIONS: DISPLACERS DERIVED FROM ASYMMETRIC PCR" NUCLEIC ACIDS RESEARCH, vol. 19, no. 9, 11 May 1991, pages 2251-2259, XP000204316 see whole doc., esp. figure 1, p.2254 ---	1-92
Y	EP 0 735 144 A (JAPAN RES DEV CORP) 2 October 1996 see the whole document --- -/--	1-92

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier document but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
"&" document member of the same patent family

Date of the actual completion of the international search

2 September 1998

Date of mailing of the international search report

30/09/1998

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2
NL - 2280 HV Rijswijk
Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,
Fax: (+31-70) 340-3016

Authorized officer

Müller, F

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 98/04819

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	KATO K: "DESCRIPTION OF THE ENTIRE MRNA POPULATION BY A 3' END CDNA FRAGMENT GENERATED BY CLASS IIS RESTRICTION ENZYMES" NUCLEIC ACIDS RESEARCH, vol. 23, no. 18, September 1995, pages 3685-3690, XP002008304 see the whole document ---	1-92
A	UNRAU P. & DEUGAU K.V.: "Non-cloning amplification of specific DNA fragments from whole genomic DNA digests using DNA 'indexers'" GENE, vol. 145, - 1994 pages 163-169, XP002054436 see the whole document ---	1-92
P,X	GUILFOYLE R.A. ET AL.: "Ligation-mediated PCR amplification of specific fragments from class-II restriction endonuclease total digest" NUCLEIC ACIDS RESEARCH, vol. 25, no. 9, - 1 May 1997 pages 1854-1858, XP002076198 see the whole document -----	1-92

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/US 98/04819

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP 0735144 A	02-10-1996	JP 2763277 B	11-06-1998
		JP 9028399 A	04-02-1997
		JP 2763278 B	11-06-1998
		JP 8322598 A	10-12-1996
		AU 692685 B	11-06-1998
		AU 5031196 A	10-10-1996
		US 5707807 A	13-01-1998
<hr/>			

Form PCT/ISA/210 (patent family annex) (July 1992)



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

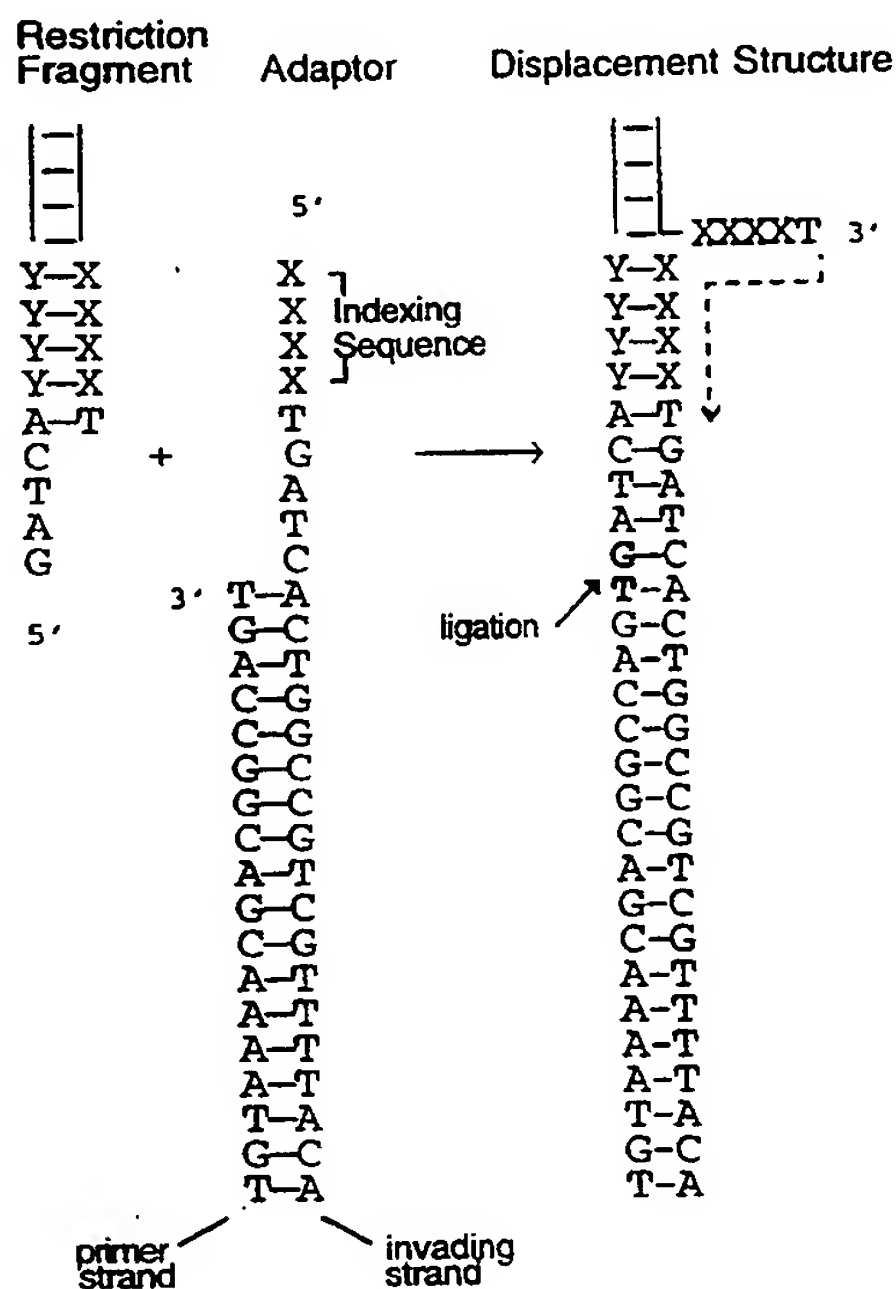
(51) International Patent Classification ⁶ : C12Q 1/68		A3	(11) International Publication Number: WO 98/40518
			(43) International Publication Date: 17 September 1998 (17.09.98)
(21) International Application Number: PCT/US98/04819			(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, GM, GW, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).
(22) International Filing Date: 11 March 1998 (11.03.98)			
(30) Priority Data: 08/815,448 11 March 1997 (11.03.97) US			
(71) Applicant (for all designated States except US): WISCONSIN ALUMNI RESEARCH FOUNDATION [US/US]; 614 North Walnut Street, P.O. Box 7365, Madison, WI 53707-7365 (US).			
(72) Inventors; and (75) Inventors/Applicants (for US only): GUILFOYLE, Richard, A. [US/US]; 19912 Gateshead Circle, Germantown, MD 20876 (US). GUO, Zhen [CN/US]; Apartment 1044, 14611 N.E. 39th Street, Bellevue, WA 98007 (US).			
(74) Agent: BERSON, Bennett, J.; Quarles & Brady, P.O. Box 2113, Madison, WI 53701-2113 (US).			

Published

With international search report.

(88) Date of publication of the international search report:
17 December 1998 (17.12.98)

(54) Title: NUCLEIC ACID INDEXING



(57) Abstract

A restriction site indexing method for selectively amplifying any fragment generated by a Class II restriction enzyme includes adaptors specific to fragment ends containing adaptor indexing sequences complementary to fragment indexing sequences near the termini of fragments generated by Class II enzyme cleavage. A method for combinatorial indexing facilitates amplification of restriction fragments whose sequence is not known. Profiling methods and other methods for characterizing polynucleotides are presented.

*(Referred to in PCT Gazette No. 8/1999, Section II)

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Latvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav Republic of Macedonia	TM	Turkmenistan
BF	Burkina Faso	GR	Greece	ML	Mali	TR	Turkey
BG	Bulgaria	HU	Hungary	MN	Mongolia	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MR	Mauritania	UA	Ukraine
BR	Brazil	IL	Israel	MW	Malawi	UG	Uganda
BY	Belarus	IS	Iceland	MX	Mexico	US	United States of America
CA	Canada	IT	Italy	NE	Niger	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NL	Netherlands	VN	Viet Nam
CG	Congo	KE	Kenya	NO	Norway	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NZ	New Zealand	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's Republic of Korea	PL	Poland		
CM	Cameroon	KR	Republic of Korea	PT	Portugal		
CN	China	KZ	Kazakstan	RO	Romania		
CU	Cuba	LC	Saint Lucia	RU	Russian Federation		
CZ	Czech Republic	LI	Liechtenstein	SD	Sudan		
DE	Germany	LK	Sri Lanka	SE	Sweden		
DK	Denmark	LR	Liberia	SG	Singapore		
EE	Estonia						

NUCLEIC ACID INDEXING

CROSS-REFERENCE TO RELATED APPLICATIONS

This application claims priority to U.S. application serial number 08/815,448 filed March 11, 1997, which is
5 incorporated herein by reference in its entirety.

BACKGROUND OF THE INVENTION

It is known in the art of molecular biology that a nucleic acid fragment lying between two identified and unique primer sequences can be amplified using the
10 polymerase chain reaction (PCR) or modifications of the PCR. PCR avoids conventional molecular cloning techniques that require the existence in nucleic acid of advantageous restriction endonuclease cleavage sites. One identified
15 shortcoming of PCR is that fragments greater than about 40 kilobase pairs between the PCR primers are only weakly amplified. It has been difficult to obtain meaningful sequence data from large genomic fragments, particularly when such fragments are resistant to traditional cloning methods. Thus, the art is seeking new methods to obtain
20 the nucleic acid sequences of long, uncharacterized regions of genetic material.

Efforts to amplify a specific DNA cleavage fragment from a population of such fragments have included methods that involve cleaving the DNA using Class IIS enzymes or
25 interrupted palindrome enzymes to form fragments having non-specific terminal 5' or 3' overhangs of various lengths (generally 2 to 5 bases). Smith, D.R., PCR Methods and Applications 2:21-27, Cold Spring Harbor Laboratory Press (1992); Unrau, P. and K. Deugau, Gene 145:163-169 (1994);
30 US Patent Number 5,508,169 (Deugau et al.); Zheleznaya, L.A. et al., Biochemistry (Moscow) 60:1037-1043 (1995). Class IIS enzymes cleave DNA asymmetrically at precise

distances from their recognition sequences. Interrupted
palindrome ("IP") enzymes cleave symmetrically between a
pair of interrupted palindromic binding sites. To amplify
the products of such cleavages, nucleic acid indexing
5 linkers, containing protruding single strands complementary
to the cohesive ends of Class IIS- or IP cleavage sites
(rather than recognition sequences) and PCR primer sites,
have been annealed and ligated to fragments generated by
Class IIS- or IP cleavage.

10 The overhangs vary in base composition, and are
determined by the locations of the enzymes' cleavage sites
in a genome. The base composition and sequence of the
overhang created after cleavage with a Class IIS or IP
enzyme cannot be predicted because the sites at which those
15 enzymes cleave DNA are determined by spatial relationship
to the recognition sequence, but are not sequence-
determined. In the methods described by Smith, Unrau,
Deugau and Zheleznaya, the unique cleavage sites generated
by Class IIS and IP enzymes determined a random sequence by
20 which fragments could be indexed. However, that is not the
case with more popular Class II enzymes that cleave within
their recognition sites and generate predictable, identical
sticky ends on each restriction fragment. Also, Unrau's
method employs temperatures that result in a problem of
25 illegitimate base pairing as well as problems with primer
dimers, where indexing fragments anneal with one another
rather with the target DNA.

What is desired is an indexing system that relies upon
fragments not generated by Class IIS or IP enzymes, and
30 which offer improved amplification specificity.

BRIEF SUMMARY OF THE INVENTION

The present invention is summarized in that
oligonucleotide adaptors for directing PCR amplification
can be engineered to efficiently and selectively hybridize
35 "fragment indexing sequences" of one or more bases
immediately adjacent to a Class II restriction enzyme

recognition sites at the termini of a nucleic acid fragment. A Class II enzyme cleaves nucleic acid within its recognition site to generate a characteristic 5' or 3' overhanging end or blunt end. The recognition site can
5 include one or more bases that do not form part of the end that results from enzymatic cleavage. When the adaptor and the nucleic acid fragment are brought together under conditions suitable for intra-strand hybridization, the invading strand of the adaptor displaces a portion of the
10 nucleic acid fragment.

Each oligonucleotide adaptor comprises a duplex portion and a single-stranded portion. The duplex portion comprises an invading strand and a complementary PCR primer strand hybridized to the invading (displacing) strand. The
15 oligonucleotide adaptors for the two termini are distinct, in that the PCR primer strands (and their complements on the invading strand) of each end adaptor are selected to specifically amplify fragments in the forward or reverse direction. The PCR primer strand, which contains the
20 sequence that is the same as that used for a PCR primer, provides a 3'-OH group that is required to join the adaptor to the restriction fragment in the method. The invading strand, which is longer than the PCR primer strand, also includes a protruding single-stranded portion that
25 comprises (1) a nucleic acid sequence that can hybridize to the characteristic overhang and (2) an adaptor indexing sequence that is perfectly complementary to the fragment indexing sequence. The adaptor indexing sequence is provided at the 5' end of the single-stranded portion of
30 the invading strand.

The invention is further summarized in that oligonucleotide adaptors of the type described can be used in a method for amplifying a restriction fragment that includes the steps of:

- 35 (a) cleaving linear or circular nucleic acid at a restriction enzyme recognition site with at least one rare-cutting Class II restriction enzyme to generate a linear

restriction fragment having a characteristic 5' or 3' overhang at each fragment terminus;

(b) hybridizing to each terminus of the fragment an end-specific oligonucleotide adaptor, thereby displacing one strand of the fragment;

(c) enzymatically ligating the restriction fragment to the primer strand to form a strand-displaced structure; and

(d) amplifying the strand-displaced structure.

The invention is further summarized in that a combinatorial degenerate mixture of oligonucleotide adaptors comprising every indexing sequence is also useful in a method for combinatorial indexing.

In a related aspect, the invention is summarized in that in a method for combinatorial indexing, genetic material cleaved with a rare-cutting enzyme produces a set of fragments for subsequent amplification. The cleaved DNA is added into an array of separate amplification reactions, where each reaction contains both an adaptor specific for one fragment indexing sequence and the degenerate combinatorial mixture of all indexing adaptors specific to the other end of the fragment. Undesired complexity in reaction processing is avoided by including both the single end-specific adaptor and the combinatorial array of adaptors in the hybridization step.

In addition to obtaining valuable sequence data from the amplified fragments, it is possible to order the fragments by generating a restriction map by performing cross-digestion using two or more different enzyme arrays. By selecting the adaptor sequence, various PCR-related methods can be employed directly on the amplification products, including PCR sequencing.

The invention is further summarized in that the adaptors are advantageously employed in methods for indexing, profiling, and characterizing polynucleotides. Adaptors can be grouped into desirable groups to acquire and analyze data gathered in the indexing, profiling and characterizing methods.

It is an object of the present invention to facilitate genetic profiling.

It is another object of the present invention to facilitate accessing and sequencing regions of the human
5 genome that are resistant to molecular cloning.

It is yet another object of the present invention to amplify nucleic acid fragments with specificity.

It is a feature of the present invention that the overhang generated by cleavage with a Class II enzyme is
10 predictable and invariant for each enzyme.

It is another feature of the present invention that the indexing sequence is separate from (not a part of) the overhang generated by restriction enzyme cleavage.

It is yet another feature of the present invention
15 that a degenerate collection of adaptors containing all possible indexing sequences is used in combination with a defined adaptor duplex to amplify unknown sequences of enzyme-cleaved nucleic acid.

It is an advantage of the present invention that the
20 methods rely upon Class II enzymes rather than the less common Class IIS and IP enzymes.

It is another advantage of the present invention that the hybridizing regions of the fragments and adaptors are longer than have been used in previous indexing systems.

25 Another advantage of the present method is the remarkable specificity with which adaptors anneal to restriction fragments when there is perfect matching between the bases of the indexing sequence and the complementary basis of the restriction fragment.

30 A fully automated PCR adaptor array strategy could bypass conventional cloning by simultaneously generating a restriction map and DNA fragments for subcloning or direct sequencing from 0.5 Mb in about one day while avoiding problems associated with so-called unclonable regions. If
35 large DNA pieces are to be mapped and sequenced, the DNA (up to about 0.5 Mb) must be purified using an existing technology such as site-specific excision (RARE, achilles

heel, PNA) or RARE-cutter restriction endonucleases (e.g., NotI or meganucleases (intron-encoded endonucleases)).

It is also possible to combine the method with conventional PCR, or to use the method in a process for
5 chromosome walking from the ends of fragments using indexers determined while preparing a restriction map.

Another application for the method is in genetic mapping to amplify fragments generated in restriction fragment length polymorphism (RFLP) analysis. Amplified
10 fragments created from such fragments would be sequence-ready and could be used directly as probes in genetic mapping. It may also be advantageous to first perform representational difference analysis (RDA) (Lisitsyn, N. et al. Science 259:946-951 (1993)) or RFLP-subtraction
15 (Rosenberg, M. et al., PNAS USA 91:6113-6117 (1994)) to reduce the complexity.

The method could also be used as an alternative to AFLP (Vos, P. et al., N. A. R. 21:4407-4414 (1995)) or arbitrarily-primed-PCR for analyzing altered gene
20 expression by differential display (Perucho, M. et al., Methods in Enzymology 254:275 (1995); Liang, Methods in Enzymology 254:304 (1995)). This method would have advantages over AP-PCR such as reduced noise and cleaner probes for gene hunting, better detection of rare messages,
25 and a requirement for a smaller number of oligonucleotides.

Other objects, advantages, and features of the present invention will become apparent upon consideration of the following detailed description taken in conjunction with the drawings.

30 BRIEF DESCRIPTION OF THE SEVERAL VIEWS OF THE DRAWINGS

Fig. 1 shows an embodiment of the restriction site indexing method of the present invention. The figure depicts one end of a restriction fragment generated by cleavage with a Class II enzyme that generates a defined 5'
35 overhang, a partially single stranded adaptor duplex and the displacement structure formed by hybridization and

ligation of the fragment and the adaptor.

Fig. 2 shows a schematic embodiment of the invention where the restriction fragment generates a defined 3' overhang.

5 Fig. 3A depicts the end-specific adaptors used in the preferred embodiment to amplify the internal BclI fragments of λ DNA.

Fig. 3B shows the degenerate set of combinatorial adaptors used in the preferred embodiment to provide a
10 proof of concept of the invention.

Fig. 4 shows the end-specific adaptors used in a method for differential display of cDNAs in accordance with the present invention.

Fig. 5 shows a strategy for amplifying 3' ends of mRNA
15 (cDNA) using adaptors of the present invention.

DETAILED DESCRIPTION OF THE INVENTION

Reference is made to Fig. 1 which illustrates an embodiment of the restriction site indexing method of the present invention. In Fig. 1, a restriction fragment
20 generated by cleavage with a Class II enzyme generates a defined 5' overhang (see left side of Fig. 1). In Fig. 2 (SEQ ID NO:27 through SEQ ID NO:31), a restriction fragment generated by cleavage with a Class II enzyme generates a defined 3' overhang (see left side of Fig. 2). When the
25 enzyme generates a 3' overhang, the longer strand can act as both invading strand and primer strand. For example, in Fig. 2, the M13 forward primer (TGTAACGACGGCCAGT) (see also, SEQ ID NO:1) is the first 18 bases of the longer strand. The 18-mer primer oligonucleotide needs to be
30 added for PCR amplification. No fill-in of the adaptor is required, as it is in the 5'-overhang case. Except as noted herein, the invention functions in the same manner when the enzyme generates a 3' overhang.

In the convention of this patent application,
35 "forward" primers are specific for the "left" end of a fragment; "reverse" primers are specific for the "right"

end of a fragment, where the fragment is presented with the 5' -> 3' strand as the top strand. As noted, a unique primer can be provided for all adaptors, if 2-strand sequencing is not desired.

- 5 Each fragment generated by cleavage of nucleic acid with a Class II restriction enzyme can be defined by a pair of fragment indexing sequences, defined as the one or more bases adjacent to the terminal recognition sites of a Class II restriction enzyme used to generate the fragment.
- 10 Accordingly, a unique pair of indexing adaptors, having the partially-singled stranded structures described herein, can hybridize to the two termini of a fragment.

Even though the characteristic overhangs at the termini are identical, the fragment indexing sequences
15 adjacent to the recognition site are not predictable; any combination of bases can reside at the indexing positions. It is noted that, because of an enzyme's cleavage strategy, one or more base pairs of the complete recognition site (e.g., in the exemplified embodiment of Fig. 1, an A-T
20 pair) can remain near the fragment terminus and should be accommodated during adaptor design.

Adjacent to the enzyme recognition site are the bases of the fragment indexing sequence, shown in Fig. 1 as X, which can be, but need not be, identical bases. In the
25 fragment, Y represents the base complementary to X at a given position. Thus, if X is A, Y can be T; if X is G, Y can be C; if X is C, Y can be G, and if X is T, Y can be A. Other recognized non-natural base pairs can also form. Because the fragment indexing sequence is not a part of the
30 recognition or cleavage sequence *per se*, neither the indexing sequence, nor its length, are limited by the choice of enzyme. This is an advantage over ligation-mediated indexing systems known in the art.

The chance that any one indexing sequence will
35 correspond to more than one terminus decreases as the indexing sequence length increases. Accordingly, it is desirable to select a preferred indexing sequence length.

The suitable size of the fragment indexing sequence will depend upon the application to which the method is put. If the goal is specific fragment amplification, greater specificity is desired so the indexing sequence should preferably be 3, 4, or 5 bases long. However, fragment fingerprinting or differential display of cDNAs can be accomplished using a preferable indexing sequence length of 1, 2, or 3 bases. An upper limit of 10 bases in the indexing sequence is contemplated.

10 By way of example only, the case of preparing adaptors for amplifying a fragment is considered. There are 64 3-base-long indexing sequences, 256 4-base-long indexing sequences, and 1024 5-base-long indexing sequences. A 4-base-long indexing sequence (256 choices) is preferred.
15 Three- or five-base-long indexing sequences could possibly be used, although if a shorter sequence were used, the selectivity would be compromised (in the sense that more fragments would be amplified per adaptor pair), and if a longer sequence were employed, sample handling becomes
20 increasingly difficult because of the array size.

It is also desirable to select a preferred nucleic acid cleavage frequency. If many fragments are generated, the likelihood that more than one fragment will be recognized by identical adaptor pairs increases. One of
25 ordinary skill will appreciate that the desired number of fragments will depend upon the application to which the method is put. If few fragments are generated, PCR amplification of longer fragments (with the accompanying art-recognized difficulties) will be required.

30 Thus a rare-cutting enzyme is preferred. In methods for restriction mapping or DNA fingerprinting, and for complex genomes, the preferred restriction enzyme used to cleave the target DNA is a 6-cutter. Five-cutters could be used, except that they are few in number and recognize
35 degenerate sequences, thereby adding to the complexity of the required adaptors. Four-cutters are thought to be unsuitable because of their abundant distribution of

cleavage sites. Enzymes cutting at sites of greater than 6 bases are also believed to be unfeasible, given their extreme rarity in the genome. On the other hand, for genomes of lower complexity, or for RNA fingerprinting (using cDNA targets) and differential display applications, 4-cutter enzymes would be suitable. Combinations of enzymes having different cleavage frequencies can be well suited for generating fragments having a certain desired average size, or for a particular target sequence.

10 A simple calculation for 6-cutters predicts that 256 individual, sequence-ready restriction fragments can be amplified from a target DNA of up to 0.5 megabases (Mb) in size. DNA of 1 Mb complexity digested with a 6-cutter enzyme, which cleaves a random sequence on average every 15 4096 base pairs, will produce 244 fragments, on average. Dividing this by 256 indexers yields about 1 amplified fragment per end-specific adaptor/combinatorial adaptor pair used. An indexing sequence would be present twice in the full library (array) of adaptors, with one contributed 20 by the end-specific adaptor and the second by the combinatorial adaptor. A fragment would be amplified twice, but at different locations in the array, and therefore a 0.5 Mb target DNA segment would be accommodated bidirectionally for isolating individually amplified 25 restriction fragments. If the target DNA is greater than 0.5 Mb, the method is still applicable using either complete digests or partial random digests such that more than one restriction fragment may be amplified per well.

 The above-noted combination furnishes the convenience 30 of easy to automate arrays of 256 members and a distribution of restriction sites that yields amplification lengths compatible with state-of-the-art PCR amplification technology.

 The center of Fig. 1 shows an indexing adaptor of the 35 type described. Indexing adaptors contain a region for PCR priming (or other function), a region complementary to a Class II restriction enzyme recognition site, and a strand-

displacement region which is complementary to the fragment indexing sequence adjacent to the recognition site on the overhang strand.

Although it is referred to herein for convenience as the PCR primer strand, the strand can comprise any sequence that is desired to be placed at a terminus of a fragment having the specified indexing sequence and can provide any desired function, for example, a restriction enzyme recognition / cleavage site, to facilitate subsequent processing of amplified fragments. Thus, the adaptors of the present invention have appreciably broader utility than for PCR amplification. If the function to be provided by the adaptor is PCR amplification, then the sequence should be unique or present in low copy number, should provide an available 3' end and should be recognized by a suitable polymerase enzyme, such as Taq or TthI polymerase. The -21M13 forward primer or the M13revP reverse primer (together, "the M13 primers") are suitable primers if the amplified fragments will be used for subsequent bi-directional sequencing. The -21M13 and M13revP primers are specific for the left and right ends of a restriction fragment, as those terms are used herein. The M13 primers, used as described herein, permit amplified fragments to be sequenced on both strands. If bi-directional sequencing is not desired, distinct primers need not be provided. For terminal fragments of linear nucleic acid molecules, a suitable amplicon-specific terminal primer can be provided in place of an adaptor if the terminal sequence is known. The sequences for amplifying the fragment can also be sequences for elongation of a template by a DNA or RNA polymerase, such as a T3 promoter, a T7 promoter, an SP6 promoter, or a sequence complementary to same.

The invading strand includes a portion complementary to the primer strand. Also, adjacent to that portion is a sequence that can hybridize to the Class II enzyme recognition site of the fragment terminus (including any residual bases near the fragment terminus that do not form

part of the overhang) to form the displacement structure shown at the right in Fig. 1. Note that although a second displacement structure, wherein the indexing sequence is displaced by the restriction fragment, could form, it is
5 not favored and is not observed, for it results in a net loss of 5 nucleotides available for annealing by the invading strand.

Strand-displaced structures of this type are described in EP-A-0 450 370 A1, Quartin et al., Biochemistry 28:8676-
10 8682 (1989), Weinstock and Wetmur, Nucleic Acids Res. 18:4207-4213 (1990), and Wong et al., Nucleic Acids Res. 19:2251-2259 (1991), which are incorporated herein by reference in their entirety, most particularly the parts relating to the formation, structure and properties of
15 strand-displaced structures useful in the present invention.

The above-noted documents describe Branch Capture Reactions ("BCR") that involve sequence-dependent attachment of a single-stranded tail to a duplex DNA, in
20 which one strand of the duplex is displaced by the single-stranded tail. The strand-displaced structure formed in BCR is akin to that of the present invention, and the parameters of formations described in the publications may be used in carrying out indexing methods of the present
25 invention. However, the documents are directed to capturing specific, individual DNA fragments from a mixture using unique sequences rather than a mixture of indexing sequences, and concern direct cloning of the captured fragments rather than indexing analysis of polynucleotides
30 in a mixture.

The documents also relate that formation of strand-displaced structures and capture of specific fragments can be facilitated by incorporating duplex-stabilizing modified bases in the capture tail. The present invention can be
35 carried out without using modified bases in the adaptor single-stranded indexing region. Nevertheless, modified bases can be employed in the present invention to stabilize

duplexes formed between an indexing adaptor and a polynucleotide to be indexed. In some circumstances, it may prove particularly advantageous to do so. Among the modified bases of this type are pyrimidines substituted with bromine at C5, particularly C5-substituted BrdC (5-bromodeoxycytidine). Also useful in this regard are 5-methyl substituted pyrimidines, particularly 5-methyldeoxycytidine.

DNA ligase efficiently joins the adaptor to the restriction fragment only if the adaptor indexing sequence is perfectly complementary to the corresponding fragment indexing sequence. Even one mismatched base in the adaptor indexing sequence will discourage efficient ligation and subsequent PCR amplification relative to a perfectly matched adaptor.

However, the hybridizing portion need not be completely complementary to the overhang, in the sense of classic Watson-Crick base pairing. A universal mismatch base analog (such as 3-nitropyrrole) could be positioned within the restriction site to elicit an effect on the indexing sequence moiety. Moreover, a string of such base analogs could be used to completely replace every base within the restriction site, so that all four indexer bases could experience enhanced discrimination and a universal adaptor could be developed for most 6-cutter restriction enzymes. This would require that the base analog or analogs incorporated not greatly affect ligase activity.

By positioning a universal base mismatch in 3 to 4 base proximity to a natural base mismatch, the T_m is lowered by up to 8°C relative to a perfect match. This discrimination enables one to amplify only fragments that perfectly match the indexing sequence provided from a digest containing many fragments. Although this can lower overall duplex stability by as much as 15°C, the enhanced discrimination would be significant for the indexing sequences. This is because discrimination is generally reduced at natural base mismatches near 3' ends, for

example, where the indexer sequences are located in the adaptor oligonucleotides.

Both positional and compositional differences may have an effect upon hybridization efficiency. It is anticipated
5 that differences in discrimination by adaptors for indexing sequences may relate to GC content, illegitimate base pairing issues, proximity to the site of ligase joining, and contiguous base stacking effects.

One or more natural base analogs (such as 5-
10 nitroindole) can also be added to the overhanging 5' end of an adaptor, if desired, to center the indexing sequence in the hybridizing portion thereby further enhance discrimination between exact and mismatched indexing sequences. The number of such bases that can be added can
15 be as long as the number of bases in the portion of the invading strand that is complementary to the restriction recognition sequence.

Improved discrimination is most apparent when the universal mismatch nucleotide is provided in either of the
20 first two positions adjacent to the indexing sequence unless the position is itself adjacent to a mismatch, which causes reduced stability. When the universal mismatch is provided any closer than three bases from the site at which subsequent ligation occurs, it is thought that the non-
25 natural base interferes with ligation efficiency and less amplified product is produced relative to that amount produced after combining the adaptor having a perfectly matched indexing sequence.

The indexing adaptor can be formed by hybridizing a
30 primer strand and an invading strand together under standard annealing conditions. A primer strand and an invading strand can be synthesized separately using oligonucleotide synthesis methods that are conventional in the art. Many oligonucleotide primers for use as primer
35 strands are readily commercially available. The M13 primers are commercially available, are in widespread use, and can be fluorescently tagged. In addition, the M13

primers have annealing temperatures that are very close to one another. This property is desirable in that both the forward and reverse amplifications can proceed with comparable efficiency under a single set of conditions. As
5 noted, the two sequencing primers need not be used if direct sequencing is not desired.

The invention can be embodied in a method for amplifying fragments of known sequence, using readily engineered adaptors having suitable adaptor indexing
10 sequences specific for both ends of the known fragment. Also, by providing combinatorial mixtures comprising all possible adaptors specific to the fragment ends, one can amplify any fragment without knowing the identity of the indexing sequence specific for either terminus. The
15 invention can also be practiced on a fragment where one end is known but the other end is unknown, by employing in the method one end-specific adaptor or amplicon-specific primer for the known fragment end and a combinatorial adaptor mixture for the other fragment end thereby permitting
20 amplification of a fragment containing known and unknown sequences, such as intron regions and flanking sequences beyond viral junctions.

The method is applicable to various targets including previously "unclonable" regions from genomic DNA, since
25 there is no need to clone such fragments to obtain useful DNA sequence. Also, large fragments can be directly cleaved and isolated from complex genomes for subsequent analysis using the method. Also, intron sequences, the sequences flanking viral integrants, can be isolated and
30 sequenced, as can terminal fragments from YAC, BAC, P1, plasmid or cosmid clones. The method can also be used to generate STS-like probes at rare-cutter restriction sites. Also, it will be possible to excise fragments surrounding regions of ambiguous sequence for further sequencing using
35 the method.

In a method embodying the present invention, a population of fragments is generated from a nucleic acid

sample by cleaving the sample with a Class II restriction enzyme. The identity of the Class II restriction enzyme is not critical, except to the extent that the sequence of the terminal overhang must be known, for preparing suitable
5 adaptors. A comprehensive list of restriction endonucleases, including Class II enzymes, in Robert and Macelis, Nucleic Acids Research 26: 338-350 (1998); see also <http://www.neb.com/REBASE> on the world wide web. When selecting a restriction enzyme and designing the respective
10 adaptors for use with that enzyme for restriction mapping or isolation of "sequence-ready" fragments, it is advantageous, but not essential, to minimize the differences in the composition of the recognition site by forming an overhang whose 4 bases are G, A, T and C. Any
15 of about 50 known Class II 6-cutters (including isoschizomers) generate 3' or 5' overhangs whose 4 bases are G, A, T and C. The available enzymes include, but are not limited to, BamHI, HindIII, AvrII, ApaLI, KpnI, SphI, NsiI, and SacI. Among these enzymes, only the outermost
20 base remaining after cleavage will vary in composition. The outermost base makes only a small and almost inconsequential contribution to the T_m for adaptor-fragment annealing. This facilitates the ligation protocol, but is not to be considered essential to the invention. This
25 design parameter also facilitates the method by helping to confine discrimination analysis to the base composition of the indexer sequences. In addition to Class II enzymes that generate four base overhangs, other enzymes that may be used effectively in the method are those that cleave
30 palindromic sequences in opposite polarity, those that leave either blunt ends or different length overhangs (e.g., not 4-base overhangs), and those that leave base compositions other than A, G, T, and C.

After cleavage, one or more pairs of partially single-
35 stranded indexing adaptors are hybridized under standard annealing conditions to the termini of one or more fragments generated by the enzyme cleavage. Each fragment

can hybridize to a single pair of adaptors. As noted above, the sequence that complements the restriction recognition sequence can include an universal mismatch to improve discrimination between adaptor indexing sequences that are perfectly-matched and imperfectly-matched to the fragment indexing sequences. *Bona fide* amplification occurs when adaptors containing perfectly-matched indexing sequences are hybridized, thus there is advantage to favoring the ability of such sequences to hybridize. Hybridization should be sufficiently strong to permit subsequent ligation of fragment termini to a pair of adaptors.

After hybridization, the gap between the primer strand and the overhanging strand of the restriction fragment is closed by treating the structure with DNA ligase under standard conditions (see Fig. 1, right side), thereby joining the overhanging strand to the primer strand. T4 ligase (NEB), thermostable Ampligase (Epicentre Technologies) ligase enzymes are suitable and have been used successfully at temperatures up to 50°C. Other ligases may also be used. Suitable ligation conditions are typical of those used in the art. The result of this step is to introduce an end-specific PCR primer (or other desired sequence) onto each end of each fragment. The primer is attached only to fragments bearing a suitable indexing sequence.

Note that during hybridization the single-stranded portion of the adaptor hybridizes to its complementary sequence on the overhang strand and displaces the fragment indexing sequence (and any residual bases of the recognition site) on the opposite strand. In the special case of a 5' terminal overhanging fragment (shown in Fig. 1), the invading strand is not covalently joined to the restriction fragment. Thus, before amplification can proceed, the displaced strand is extended from its 3'-end by polymerase in the first thermal cycle to regenerate a template complementary to the PCR primer. This extension

step is not required if the termini have 3' overhangs (Fig. 2).

Fragments can be amplified using standard PCR reactions such as those described in the Example. In the preferred embodiment, one set of PCR conditions is suitable to amplify fragments of most sizes, although it may be necessary in certain cases to adjust the PCR conditions in accordance with the abilities of one skilled in the art to amplify a particular fragment. PCR protocols can be varied to accommodate particular sequences and primers. One skilled in the art will appreciate that certain modifications to the PCR protocols may be required to amplify particular fragments. Such modifications may include varying primer length, adjusting magnesium concentration, adjusting thermal cycle time, adjusting the annealing temperature and the like. It is necessary to add additional primer before amplifying. One skilled in the art will also appreciate, for example, that so-called long distance PCR conditions can be employed to amplify fragments greater than about 3 kb, although success under such conditions cannot be assured, as such protocols are still under development by the art.

Occasional false amplifications may be observed if a particular indexing sequence forms a more stable mismatch when hybridizing with the restriction fragment. However, one having ordinary skill can determine hybridization conditions under which such mismatches are not observed and do not give rise to amplification products.

In another aspect, the invention is also a system for combinatorial indexing. Combinatorial indexing is advantageously employed when seeking to separately amplify restriction fragments where the index sequence of each fragment terminus is not known. It will be appreciated that by providing every adaptor specific to both ends, all fragments generated by enzyme cleavage can be amplified, even without *a priori* knowledge of the sequence. In the method described above, by contrast, each fragment terminus

has an indexing sequence selected from one of the possible indexing sequences (e.g., 1 of 256 possible 4-base-long indexing sequences). The unique combination of indexing sequences corresponding to the termini of an unknown
5 fragment is one of 65,536 possible pairwise combinations of 256 left-end-specific indexing sequences and 256 right-end-specific indexing sequences.

Such a large array of possible combinations is methodologically impractical (even if automated), but would
10 be necessary to recover all possible restriction fragments that could be generated from total digestion of a larger DNA. Even if automated, the handling of such a large array would be formidable. However, the size of the array can be reduced to 256 simply by providing in each reaction a
15 single unique left or right end-specific adaptor along with a degenerate mixture of 256 adaptors corresponding to the second fragment end. Such mixtures are referred to herein as a "combinatorial adaptor" or a "C-adaptor." The C-adaptor mixture can be made in a single
20 oligodeoxynucleotide synthesis process by providing all 4 nucleotides (A, G, C, T) at each adaptor indexing sequence position.

Reducing base-pairing specificity provides a way to control the number of possibilities, by combining adaptors
25 into sets or by substituting modified bases that can pair with more than one base, or both. For instance, Py- and Pu-specific bases would have a 1 in 2 probability of pairing with each base in a random sequence, whereas any of A, C, G and T have a 1 in 4 possibility. Consequently, for
30 $n=2$, for example, using any of A, C, G or T for position 1 and either Py or Pu for position 2 produces only 8 possibilities rather than 16. Likewise, for $n=3$, using Py or Pu in place of A, C, G or T for any one of the three positions provides 32 instead of 64 "sequences." The
35 ability to control the number of indexing sequences in this way may be used to reduce the number of reactions and separations needed to index a given polynucleotide

population.

The specificity at each position of an indexing sequence may be provided by A, C, G, T or modified bases. Reduced specificity may be provided by mixing indexing
5 sequences or by synthesizing oligonucleotides with two or more bases in one or positions of the indexing sequences. Thus, N can be provided by mixing indexing sequence having each of A, C, G and T at the given position of N in the indexing sequence, or it can be provided by synthesizing an
10 oligonucleotide with a mixture of A, C, G and T at the given position for N. N-specific positions can also be provided using modified bases (such as those described elsewhere herein).

A PCR reaction would yield an amplified fragment only
15 when it contains both the end-specific indexing sequence as well as to one of the indexing sequences in the combinatorial adaptor. In 256 separate ligation/PCR reactions, the probability is that each reaction amplifies a single, sequence-ready restriction fragment. Although
20 the invention is practiced by providing an adaptor specific to each end when 2-strand direct sequencing of the PCR products is desired, the invention can also be practiced by providing a single primer for both ends. The invention can also be practiced using a single adaptor, if PCR
25 amplification is not desired. For example, a restriction fragment and a primer strand tagged with a reporter molecule can be annealed to a surface-bound invading strand, without subsequent ligation. The restriction fragment will anneal to the invading strand where there is
30 correspondence between the adaptor- and fragment indexing sequences. The primer strand will also anneal to the invading strand. After annealing, unbound restriction fragments can be washed away. Interstrand base stacking interactions between the tagged primer strand and the
35 restriction fragment will keep the primer strand annealed only where the fragment corresponds to the invading strand indexing sequence. This can facilitate specific detection

of restriction fragments of interest. When used in this manner, the invention provides a method for ordering fragments in a clone.

To map the order of fragments, several independent
5 arrays are analyzed as described using adaptors specific for different restriction enzymes and then the product of each array can be cross-digested with the enzymes of the other digestions. The products of those cross-digestions can be separated by electrophoresis and a standard
10 restriction map can be produced for any nucleic acid fragment.

Ligation-mediated indexing using class-II enzymes can be applied to RNA fingerprinting in a way similar to that described for class-IIS enzymes (Kato, K. NAR, 24:394-395
15 (1996), incorporated herein by reference). A particular application in this regard would be for functional identification of genes by differential cDNA display. Kato and others proposed that an indexing approach could offer several advantages over the more commonly used "arbitrarily
20 primed PCR" (Liang, P. and Pardee, A.B. Science, 257:967-971 (1992), incorporated herein by reference) for this purpose, including (a) obtaining more coding regions, (b) allowing lower redundancy, and (c) detecting rare messages more efficiently.

25 An important aspect of such a fingerprinting application is the ability to adequately resolve the fragments generated. For example, differentiated or neoplastic somatic cells have a messenger RNA complexity on the order of 20×10^6 . Using a pair of 4-cutter
30 restriction enzymes to digest cDNA, fragments are obtained that should, on average, be <200 bp in size. A given message will be represented by numerous non-overlapping fragments specifically amplified using adaptors with 4-nucleotide indexing sequences. The fingerprint of the
35 256 fragment subclasses generated can be well resolved on a polyacrylamide gel.

The order of the fragments for a given message can be

determined either by (a) restriction mapping and/or sequencing the clone(s) from an appropriate cDNA library that cross-hybridize to the amplified fragments, or (b) amplifying the cDNA using the identified message-specific indexing adaptors in conjunction with primers which can access the 5'- and/or 3'-end of the message, and then restriction mapping and/or sequencing. As examples, the 5'-end of an mRNA can be located after preparing the cDNA using CapFinder technology (Clontech); the 3'-end of an mRNA can be accessed using oligo-dT primers as described by Liang and Pardee or oligo-dT coupled with a different or universal primer.

Single-enzyme strategies could also be used to obtain RNA fingerprints using indexers for class-II enzymes. Indexing can be confined to one of the cleaving enzymes if the second cleaving enzyme generates a constant, defined end. These strategies would target either the 5'-proximal or 3'-proximal restriction fragments of the cDNA. The cDNA could be cut with a single 4-cutter, ligated to the indexing adaptors containing a universal primer, and then PCR amplified by using either a CapFinder or oligo-dT associated primer. These approaches would yield less complex fingerprints than the double-enzyme approach, but would be biased toward detecting fewer coding regions and more untranslated regions (UTRs). However, UTRs represent excellent signatures for identifying unique messages.

Different strategies could be adopted to reduce array size and, therefore, sample handling. One strategy could utilize the combinatorial adaptors. Instead of using 256 single-end adaptors, adaptors could be pooled in several combinatorial mixtures which represent subclasses of the complete library (e.g. 4 pools x 64; 16 pools x 16, etc.). (A pooled subclass could also be synthesized as a degenerate oligo). The complexity of the banding pattern (per pool) will decrease as the number of pools increases. In another strategy, 3-nucleotide indexing sequences could be utilized. The size of a 3-nucleotide indexing sequence

library would be 64. However, because trinucleotide frequencies are higher than tetranucleotide frequencies in a given genome, a more complex banding pattern is expected.

Another application for the method is genetic
5 profiling, including DNA fingerprinting and RNA fingerprinting. A particularly useful DNA fingerprinting application would be detecting restriction fragment length polymorphisms. These RFLPs detect polymorphic sequences distributed throughout a genome and serve as useful markers
10 for genetic linkage mapping.

Traditional RFLP analysis required hybridizing probes of known sequence to genomic DNA digested with various restriction enzymes. However, newer methods do not require any prior probe characterizations and can be
15 applied to the fingerprinting of genomes of any complexity. These newer methods include random amplified polymorphic DNA (RAPD), DNA amplification fingerprinting (DAF), arbitrarily-primed PCR (AP-PCR), and amplified fragment length polymorphism (AFLP). In each of these methods,
20 except AFLP, random genomic DNA fragments are amplified using by arbitrarily selected primers to generate fragment patterns for any DNA without prior knowledge of its sequence. AFLP (Vos, P. et al., N.A.R. 21:4407-4414 (1995)) resembles RFLP (Bostein, D. et al., Am. J. Hum.
25 Genet. 32: 314-331), except insofar as restriction fragments are detected by PCR rather than Southern hybridization. Also, AFLP displays the presence or absence of fragments rather than their size differences. The adaptors that are ligated to the digested DNA in AFLP
30 analysis include sets of generic PCR primers, thereby permitting the sequences which reside adjacent to the restriction sites to be queried. In AFLP, fingerprints of varying complexity can be obtained by adjusting the enzymes and primer sets employed.

35 AFLP can also be used for RNA fingerprinting to detect and monitor differential gene expression, starting with different double-stranded cDNA samples for a given

comparative analysis (Money, T., et al. N.A.R. 24:2616-2617). Regardless of the source of DNA, however, a major disadvantage of this method is that it requires many variations in adaptor and/or PCR primer designs. This, in turn, demands that PCR conditions be optimized for each selected set of primers. Therefore, the AFLP technique is not highly amenable to formatting for streamlined multi-sample processing.

In the present invention, as in the AFLP method, fragments are queried at sequences located next to their sites. In the case of the present invention, however, an adaptor invading strand, rather than PCR primers, is used to interrogate those sequences. The adaptors can contain combinations or permutations of indexing sequences that anneal by strand-displacement to their corresponding polynucleotides. Indexing adaptors of the present invention can contain one primer sequence (pair) for amplifying the polynucleotide after the adaptor is ligated.

Therefore, only one set of PCR conditions need be found for all fragments amplified, without regard to the enzyme or indexing sequences employed. Furthermore, the indexing specificity is relatively insensitive to changes in temperature, time, and ligase concentration conditions used in ligating the adaptors to the polynucleotides (results not shown). This is not unexpected since it is known that the annealing reaction during branch migration at the termini of fragments is very rapid and efficient (Quartin, R.S., et al., Biochemistry 28:8676-8682).

Taken together, these advantages mean that no variations in adaptor design or PCR conditions is intrinsically necessary, unlike in AFLP, making the method highly amenable automation and high throughput.

Genetic profiling using the present invention can be carried out using a variety of strategies, for both DNA and RNA fingerprinting. For RFLP analysis, profiles would be expected to show only the presence of new fragments or the absence of fragments resulting from mutations or sequence

variations in a restriction fragment and/or an indexing sequence. The fragments created to score RFLPs would be generated using restriction enzymes that cut at frequencies amenable to PCR amplification and display by gel electrophoresis.

An RFLP strategy is chosen based on the complexity of the genomic DNA interrogated as well as the desired complexity of the fingerprint. As in the AFLP method, important variables include the character of class-II enzymes used (e.g., 4-cutter, 6-cutter) and the indexing sequencing length (e.g., 2, 3, or 4 nucleotides). For both RNA and DNA profiling, a given approach could utilize mixtures of strand-displacing adaptors and sticky-end adaptors, such as is used in an RNA fingerprinting approach for displaying differentially-expressed sequences that represent full length messages (see Examples). Alternatively, a single enzyme-adaptor set could be ligated to bring in only one PCR primer, the other being provided by a known sequence located elsewhere in the restriction fragment. This approach was used for the gene expression profiling of restriction fragments derived from the 3'-ends of mRNA in a manner resembling that described by Prashar and Weismann (U.S. Patent No. 5,712,126, incorporated herein by reference in its entirety; see also PNAS USA 93: 659-663 (1996) and see Examples).

Unlike the widely used arbitrarily primed PCR method (Perucho, M. et al., Methods in Enzymol. 254: 275 (1995) and Liang, Methods in Enzymol. 254:304 (1995), the present invention is a form of "ligation-mediated PCR" that more efficiently detects low-abundance messages by significantly reducing the redundancy of amplified products. This is so because arbitrarily selected primers randomly amplify cDNA sequences in their entirety whereas in a ligation-mediated approach, a single pair of primers (brought in by adaptors) amplify specific portions of a message.

Adaptor primer sequences can serve least four purposes: (1) amplifying by PCR to generate profiles, (2) re-amplifying specific bands by PCR for isolating and subcloning, (3) re-amplifying specific bands for use as a direct sequencing template, and (4) generating DNA or RNA probes by PCR or by in vitro transcription, respectively. A variety of strategies can be employed for designing adaptor primer sequences. For PCR applications, the primer sequences are preferably designed such that their T_m 's closely match one another, and so that the primer lengths accommodate the type of PCR reaction employed. Longer sequences may be desired, for example, to enable two-step thermal cycling, touchdown PCR, or long-distance PCR conditions. In certain cases, some sequences, such as the T7, T3, and SP6 promoter sequences, could be used for all four applications.

It may be desirable to have more than one primer sequence per adaptor, to accommodate a custom designed utility for more than one function. Resulting increases in adaptor size are not expected to significantly change the efficiency of ligation to the restriction fragments. Using primer sequences built into the adaptors, fragments isolated from fingerprints of genomic DNA or mRNA can be re-amplified and sequenced to serve as sources of genetic mapping probes or "expressed sequence tags" (EST), respectively. For DNA profiling, it may be advantageous to first perform a subtractive technique to reduce complexity, such as representational difference analysis (RDA) (Lisitsyn, N. et al., Science 259: 946-951 (1993) or "RFLP-subtraction" (Rosenberg, M. et al., PNAS USA 91:6113-6117 (1994)). For RNA profiling, it may also be advantageous to first perform a subtractive technique to enrich for differentially expressed genes (for example, see Ariazi, e. and Gould, M. J. Biol. Chem., 271:29286-29294 (1996)).

A targeted amplification strategy similar to that employed by U.S. Patent No. 5,712,126 (Prashar and

Weissman), incorporated herein by reference in its entirety, can also be used to amplify 3'-end restriction fragments of cDNAs to generate "fingerprints" or "expression profiles" from which bands can be recovered for
5 sequence analysis and EST production. These fragments contain mostly untranslated sequences, which can serve as unique identifiers for messenger RNAs. These sequences are also useful for creating and searching EST databases.

By using strand-displacement adaptors in conjunction
10 with enzymes that cut relatively frequently (e.g., 4-cutter class-II enzymes), the present invention will achieve significantly greater gene coverage than can be obtained with the Prashar and Weissman technology, which typically employs 6-cutter class-II enzymes. While U.S. Patent No.
15 5,712,126 can require up to 55 6-cutters to obtain >95% gene coverage for 3'-fragments up to 400 bp in size, it is expected that >99.9% gene coverage can be obtained using only four 4-cutters in combination with tetranucleotide indexing sequences.

20 The strategy to generate expression profiles and ESTs in this manner is as follows:

- (1) make double-stranded cDNA from total RNA or polyA+ RNA using anchored oligo-dT/heel sequence;
- (2) digest cDNA with a 4-cutter to produce
25 polynucleotide fragments;
- (3) ligate fragments to strand-displacement adaptor containing restriction site and indexing sequence;
- (4) PCR amplify fragments using primers complementary to adaptor and anchored heel sequence, where one primer is
30 distinguishable, e.g., includes a radiolabel, fluorescent dye label, or infrared dye label;
- (5) separate the amplification products, e.g., on a denaturing polyacrylamide gel;
- (6) detect the distinguishable products by, for
35 example, autoradiography (for radioisotopic labeling), fluorimaging (for fluorescent dye labelling) or IR-imaging (for infrared dye labelling);

(7) excise bands of interest (with optional re-amplification step), and determine their nucleic acid sequences; and

(8) search databases and analyze sequences.

5 Genes that are differentially expressed can be profiled and recovered by using the above strategy on samples representing different cellular states such as (a) normal vs. diseased, (b) infected vs. uninfected, (c) developing vs. adult, (d) drug treated vs. untreated, and
10 the like. Profiles are preferably displayed by running PCR products side-by-side on a denaturing polyacrylamide gel to readily observe fragments that represent genes of unchanged or altered expression. The profiling aspect of the invention can be advantageously employed in a search for
15 novel pharmaceuticals that, for example, promote or inhibit mRNA expression by cells in a particular state. In particular, characteristic reference, average or diagnostic profiles can be established for sets of cells that exhibit differential mRNA expression.

20 The most stable linkages between adaptors and fragments will likely be obtained using restriction endonucleases that generate the longest possible overhangs (Weinstock, *supra*). In the case of 4-cutters, these would be tetranucleotide overhangs such as those generated by
25 Sau3AI (DpnII) and Tsp509 I for 5'-overhangs, and Tai I and Cha I for 3'-overhangs.

Examples

Amplification

The feasibility of the specific amplification method
30 described herein was tested using N⁶-methyladenine-free bacteriophage λ DNA (48502 base pairs, New England Biolabs, Beverly, MA) as the model amplicon system and BclI, a 6-cutter, as the model Class II restriction endonuclease. Enzyme digestions were performed in the supplier's buffer
35 at 37 °C for two hours with 20 U of BclI in a volume of 100

μ l. BclI cuts the λ genome eight times, producing nine fragments that share the same 5'-overhang sequence, 5'-GATC. BclI was chosen because of the broad range of fragment sizes that the enzyme generates: 517, 560, 1576, 2684, 4459, 4623, 6330, 8844, and 18909 base pairs. The terminal fragments are 560 and 8844 base pairs. The terminal fragments include a BclI cut site at one end and the genome terminus at the other. Unique oligonucleotide primers were used to amplify the terminal λ fragments.

10 Since the entire nucleic acid sequence of the λ genome is known, adaptors were produced containing only the required adaptor indexing sequences. In the adaptors, the primer strand was either an M13 sequencing primer or M13 reverse sequencing primer, depending upon which end of the
15 fragment it was specific for. Terminal primers were provided for the terminal fragments. The invading strand comprised, in 5' to 3' order, a 4-base-long indexing sequence, a 5-base-long sequence complementary to the BclI recognition site, and a portion fully and perfectly
20 complementary to the primer strand. The primer strand and the invading strand were prepared by conventional oligonucleotide synthesis, were purified on Sep-Pak C18 cartridges and were annealed at a concentration of 12.8 μ M of each primer in 50 mM tris-HCl, pH 7.8 at 85°C. The
25 oligonucleotides were allowed to anneal by slow cooling to room temperature.

The end-specific indexing sequences used to amplify particular λ BclI fragments are shown in Fig. 3A (SEQ ID NO:1 through SEQ ID NO:20). The end-specific adaptors that
30 corresponded to the left (L) and right (R) ends of the fragments used the -21M13 (forward) and M13RevP (reverse) universal primer sequences, respectively. For each end, the primer strand is shown once and each partially-complementary end-specific invading strand is shown. The
35 indexing sequences specific to each fragment end are shown in bold and the BclI site that remains after cleavage is underlined.

Once the adaptors were prepared, the BclI fragments were individually amplified from the total BclI digest as follows:

(a) 5 μ g of N⁶-methyladenine-free λ DNA (New England Biolabs, Beverly, MA) was digested at 37°C for 2 hours with 20 units of BclI in a volume of 100 μ l using the manufacturer's (NEB) buffer;

(b) 15 ng of digested λ DNA were combined with left and right adaptor pairs corresponding to a particular restriction fragment in NEB 1X ligase buffer for 5 minutes at 40°C (each ligation contained 25 pmols of single end adaptor pairs, in equal amounts. For the right end of the genome, λ -specific primer CGTAACCTGTCGGATCAC (SEQ ID NO:21) was used. To amplify the left end of the genome (8848L), λ -specific oligonucleotide CGCGGGTTTTCGCTATTT (SEQ ID NO:22) was used);

(c) 800 units of NEB T4 DNA ligase were added and the reactions were incubated for 20 minutes at 40°C and were stopped by heating to 65°C for 15 minutes;

(d) 1.5 ng of λ DNA were transferred to 100 μ l PCR reactions. All PCR reactions were performed with the XL-PCR kit (Perkin-Elmer, Applied Biosystems Division, Foster City, CA), using 2 μ l (4 units) of rTth DNA polymerase. The PCR reactions included 1.1 mM magnesium acetate (1 mM MgCl₂ carried over from the ligase reaction), except the amplification of the 4,459 base pair BclI fragment from λ DNA which included 1.65 μ l of magnesium acetate to obtain robust and specific amplification from its combinatorial adaptor. The specific products could also be obtained using 0.55 mM magnesium acetate. All PCR reactions contained 10 pmols of appropriate primer oligonucleotides. PCR was performed in the PTC-200 DNA engine (MJ Research, Watertown, MA) using the following thermal cycling profile: 95°C for 1.5 minutes followed by 30 cycles of 94°C for 40 seconds, 55°C for 40 seconds, 72°C for 5 minutes.

Treatment with 3'-to-5' exonuclease activity of Vent polymerase was important for increasing the yields of the

PCR products obtained with rTth polymerase.

(e) 20 μ l were loaded on 0.8% agarose gels containing 0.5 μ g per μ l ethidium bromide. Specific bands were observed upon electrophoresis.

5 No reactant removal or product purifications were required between steps, making the overall procedure amenable to automation. In some conditions, it may be advantageous, but not absolutely necessary, to purify fragment-bound adaptors away from unligated adaptors or
10 fragments. A solid-phase purification step can be included. However, the need for such a solid-phase purification has not been observed.

When the appropriate left/right adaptor pairs or terminal/left or right adaptor pairs were used, eight of
15 the nine BclI fragments of λ DNA were selectively and specifically amplified. Under the conditions described, specific amplification of the 18909 base pair fragment was not observed, although the fragment was observed with additional non-specific fragments, including the 6.3K,
20 4.6K, 4.4K, 2.6K and 1.5K lambda fragments. These fragments were amplified at least in part because a longer polymerase extension time was required just to detect the 18909 base fragment. In this case, fragments arising from rare non-specific ligation events are amplified to a
25 greater extent. However, when 3-nitropyrrole was incorporated into the restriction site of the adaptor, all of the extra bands were eliminated. The suppression of the nonspecific fragments was optimal when the 3-nitropyrrole was positioned in the middle of the 9-nucleotide protruding
30 single-strand region of each of the 18.9K-specific adaptors.

It is possible to achieve good discrimination among the adaptor pairs tested. Where non-targeted restriction fragments were co-amplified along with the desired product,
35 the extra amplification can be explained by homology in some indexing sequence positions and the potential for stable mis-match duplex formation in other indexing

sequence positions. Few non-specific products that did not co-migrate with the restriction fragments were observed.

Combinatorial indexing

To demonstrate the utility of the method employing combinatorial adaptors, two sets of combinatorial primers were prepared, as is shown in Fig. 3B. The "combo-FP" adaptor included the -21M13 primer hybridized to the indicated C-adaptors, where N at each position indicated in the adaptor represents a population of all four nucleotides at that position. Thus, each mixture of combinatorial adaptors included 256 different adaptors. Likewise, the "combo-RP" adaptor set included the M13revP primer hybridized to the indicated set of invading strands where N is all four nucleotides at each position.

To amplify various fragments of BclI-cut λ DNA, the following amounts of the indicated end-specific adaptors (or primers in the case of the terminal fragments) were combined with the indicated amounts of combo-FP or combo-RP mixtures.

20

Table I

Fragment to be amplified (bp)	Right adaptors (pmol)	Left adaptors (pmol)	Combo-FP	
			mix (pmol)	mix
517				
560	10 (560R*)	---	0.0025	---
1576	25	---	0.5	---
2684	25	---	0.25	---
4459	25	---	25	---
4623	25 pmol	---	25	---
6330	---	---	---	---
8848	---	10 (8848L*)	---	---
0.0025				

*Primer only (in PCR reaction)

Specific amplification of fragments having the expected fragment length were observed by polyacrylamide gel electrophoresis, thus indicating that desired fragments can be amplified by providing an adaptor specific for one

end of a desired fragment and a mixture of adaptors containing an adaptor specific for the indexing sequence at the other end of the fragment. It is of note that no purification was required prior to PCR amplification to
5 remove ligation reactants or intermediate products.

Specific fragment amplification was driven predominantly by the end-specific adaptor ligated at one end. That is because when the end-specific adaptor and C-adaptors are provided at equimolar amounts, the relative
10 concentration of a single indexing sequence in the combinatorial mixture is only 1/256 as great as the amount of the end-specific adaptor, thereby favoring more efficient ligation of the more prevalent adaptor.

In additional tests, it was shown that specific
15 fragments were amplified from the total BclI- λ DNA digest over a range of asymmetric end-specific:C-adaptor concentration ratios. The ratios of end-specific adaptors:C-adaptors was varied from 1:1 to 100:1. An additional hundred-fold dilution of the combinatorial
20 adaptor yielded the most specific λ terminal fragment amplifications.

Amplification from genomic polynucleotides

To demonstrate that specific amplification can be accomplished in the presence of a more complex genome, *E. coli* DNA containing λ c1857Sam7dam⁻ lysogen (NEB) was used
25 as the amplification target. This more complex genome (4.7 Mb) has 1,604 BclI sites, 200 times as many as λ DNA. Despite this increase in target complexity, λ BclI fragments could still be specifically amplified using the
30 adaptors tested.

Eighteen μ g of the λ lysogen DNA was digested with BclI. Twenty five pmol (each) of left and right adaptors were used to amplify the 517, 1576, and 2684 bp fragments. Subsequent dilutions and reactions were performed as
35 described above for λ DNA.

Although the concept has been demonstrated using known

DNA, it is equally applicable to unknown DNA targets excised directly from the genome. Using the method, a DNA fragment that maps between two STS markers can be obtained. At least two 6-cutter arrays will be used in conjunction
5 with combinatorial indexing to obtain a complete restriction map of the selected fragment and the production of contigs. PCR amplification products produced from each array will be subjected to agarose gel electrophoresis to acquire fragment length information.

10 RNA fingerprinting

RNA fingerprinting using adaptors for class-II enzymes was tested for the differential display of cDNA from rat mammary carcinomas, untreated or treated with perillyl alcohol (PA) which is a monoterpene used for
15 chemoprevention and chemotherapy (Crowell, P.L. and Gould, M.N. Crit. Rev. Oncog., 5L:1-22 (1994), incorporated herein by reference). cDNA from treated and untreated tumors (at half-regression) was prepared by and according to Ariazi, E. and Gould, M. (J. Biol. Chem., 271:29286-29294 (1996),
20 incorporated herein by reference).

In a preliminary study, DpnII (GATC) and NlaIII (CATG) were used as the cleavage enzymes. DpnII provides indexing sequences next to its 5'-overhang and NlaIII provides a defined 3'-overhang for a cohesive end adaptor. Because a
25 DpnII site will not anneal with an NlaIII site, fragment chimeras are minimized and primer-dimer formation during PCR is eliminated. As is shown in Figure 4, the NlaIII adaptor contains the M13 reverse primer sequence and the DpnII adaptors contain the M13 forward primer sequence.
30 For this study, four 4-nucleotide indexing sequences were used (Fig. 4, SEQ ID NO:1 and SEQ ID NO:23 through SEQ ID NO:28). The adaptors were designed such that the chance of forming stable mismatches was minimized according to the observations of Ebel et al., Biochemistry 31:12083-12086
35 (1992), incorporated herein by reference.

A suitable protocol for generating fingerprints was as

follows. Note that if the enzyme cleavage buffers are compatible with one another, the cleavages can be accomplished in a double digestion.

- 5 (1) digest 0.5 μ g cDNA (-/+ PA treatment) with NlaIII;
- (2) clean-up*, elute in water;
- (3) join NlaIII adaptor (25 pmol) with 800U T4 DNA ligase at 37°C;
- (4) clean-up, elute in water;
- 10 (5) digest with Dpn II;
- (6) clean-up, elute in water;
- (7) split cDNA four ways (125 ng ea.) and join Dpn II adaptors (25 pmol) with 800U T4 DNA ligase at 40°C;
- 15 (8) use Klentaq (Advantage cDNA PCR kit, Clontech, Palo Alto, CA) to amplify 5 ng of ligated DNA using 25 pmol ea. of the -21M13 and M13rev primers;
- (9) run aliquots on 5% polyacrylamide electrophoresis gels; stain with Sybr Green I (Molecular Probes, Eugene, OR) to separate and visualize a characteristic pattern for amplified fragments;
- 20 (10) visualize by UV transillumination or laser scanning (Fluorimager 575, Molecular Dynamics, Sunnyvale, CA)
- 25 * each clean-up step used Qiaquick spin column (Qiagen, Chatsworth, CA) to remove enzymes, buffers and/or unligated adaptors

For the two 4-cutter approach, an average expected
 30 number of amplified products per gel lane (i.e. per indexer) was estimated by $(20 \times 106/512)/256$, or approximately 150, assuming a perfectly random distribution of sites and a perfectly random sequence of nucleotides in the total cDNA. However, because the sequences are not
 35 random in nature, fragment size range varies. For the 4 indexing adaptors tested, the size of the observed amplified fragments ranged from about 50 bp to about 300 -

500 bp. The bands were well separated and indicated a quasi-random distribution of restriction sites useful for fingerprinting and probe isolations. The fingerprints observed were highly reproducible for a given set of thermal cycling parameters and yielded differentially expressed bands indicating both up-regulation and down-regulation after PA treatment (confirmed by varying the amount of template in the PCR). The sensitivity of the assay was high, detecting as little as 2-3 fold changes in the levels of some differentially expressed bands. However, to distinguish truly differentially expressed bands from false positives, it would typically be necessary to re-amplify a band and use it as a probe against Northern blots.

15 Amplification of mRNA/cDNA 3' ends

The 3'-end-targeted amplification strategy employing the adaptors of the present invention was tested on Sau3AI digested cDNA prepared from resting and activated human (Jurkat) T-lymphocytes. Activated Jurkat T cells are known to contain highly elevated levels of interleukin-2 (IL-2) mRNA. A 40-mer oligonucleotide (CAGGGTAGACGACGCTACGC(T₁₈)AT; SEQ ID NO:32) was used as an anchor primer for cDNA synthesis and PCR primer in the fragment amplification step. In SEQ ID NO:32, CAGGGTAGACGACGCTACGC is the "heel" sequence, T18 is an 18-mer oligo-dT portion that can anneal to the poly-A tail of messenger RNAs, and AT is the dinucleotide anchor sequence. AT was chosen because its complement is contained in interleukin-2 (IL-2) mRNA. The 3'-proximal Sau3AI site of IL-2 cDNA is located 142 bp from the poly-A. The indexing sequence adjacent to the 3'-proximal Sau3AI site is AAAA on the anti-sense strand of the restriction fragment.

An adaptor was prepared by annealing an 18-mer oligonucleotide (TGTAACGACGGCCAGT; SEQ ID NO:1) corresponding to the M13 forward primer sequence and a

26-mer invading strand that contained the M13 sequence complementary to the forward primer sequence, the Sau3AI tetranucleotide overhang and the AAAA indexing sequence. Fig. 5 depicts the strand displacement structure formed by ligating the adaptor to the IL-2 Sau3AI fragment as well as the primers used for PCR. Fig. 5 also shows eight indexing sequences tested (IS-1 through IS-8) besides that for IL-2 (IS-9). An IL-2 specific fragment of 200 bp is amplified (26 bp + 40 bp + 142 bp - 8 bp, where 8 bp is the overlap between adaptor and fragment).

Total RNA was prepared, cDNA was synthesized, adaptors were formed, polynucleotides were digested, ligated and amplified using Ampligase Gold (ABI-Perkin Elmer) as described in U.S. Patent 5,712,126, except that the restriction enzyme and adaptors of Prashar and Weissman were substituted by Sau3AI and the indexing adaptors, respectively. For detecting the amplified fragments, the M13 forward primer was end-labeled with ^{32}P using polynucleotide kinase according to a standard protocol. Amplified restriction fragments were separated by electrophoresis on an 8M urea-6% polyacrylamide denaturing gel, and an autoradiograph was obtained by exposing the dried gel to X-ray film for approximately 16 hours.

The nine fingerprints observed on the autoradiograph are non-overlapping (i.e., share no apparent bands) and contain fragments representing differentially expressed genes. Base-pairing specificity within the indexing sequence was determined by re-amplifying, subcloning and sequencing five fragments excised from each of the nine fingerprints. No base pair mismatches were observed, indicating 100% specificity for 40 fragments that were targeted (5 fragments did not yield readable sequence in this experiment). Less than 20% non-specificity was observed in the systemic background, that is only in non-targeted cDNA fragments revealed only in the fragment library subclones. Close to the number of expected fragments (see table below) were observed, strongly

suggesting that gene coverage efficiency is high under the ligation and PCR reaction employed.

Furthermore, the fingerprints were consistently reproducible with respect to band patterns and signals, and 5 identical fingerprints were observed by fluorescent imaging when a tetramethylrhodamine (TAMRA)-labeled M13 primer was substituted for the ^{32}P -labeled M13 primer. From sixty nonredundant sequences analyzed from the nine adaptor profiles, 34 matches with human genes were found in the 10 Genbank-primate database (5 differentially expressed, including IL-2), 22 matches in the gb-EST database were found (2 differentially expressed), and four fragments showed no matches (1 differentially expressed) and represent novel genes.

15 In the preceding example, simple, well-resolved fingerprints (15-20 fragments per lane) are generated by using a dinucleotide anchor primer in combination with tetranucleotide indexing sequences. Although this combination yields close to the theoretical number of 20 expected 3'-fragments (~10), the number of ligation reactions to achieved 99% gene coverage is extremely large, and for 4 enzymes is [256 adaptors] x [12 N_1N_2 -oligo(dT) primers] x 4 = 10,240. This would be a formidable task even if automated.

25

Table 2

Parsing Reduction Strategies

	Strategy	5'-end		3'-end		# expected frags/lane	# ligations (4-enzs)
		IS length	set				
30	I	4	256	N_1N_2 (x12)	10	10,240	
	II			N_1 (x 3)	40	2,560	
	III			N_0	120	853	
	IV	3	64	N_1N_2	40	3,072	
	V			N_1	160	768	
35	VI			N_0	480	256	

Table 2 shows six parsing reduction strategies that could be employed to reduce the number of reactions to a

manageable size. These strategies do not consider the use of "combinatorial mixtures" of base-pairing specificity, which could further reduce the total number of reactions required. In Table 2, parsing reduction can be targeted to one or both ends of the restriction fragments by reducing the number of indexing bases (5'-end) from four to three in the ligation step, or oligo-dT anchor bases (3'-end) from two (N_1N_2) to one (N_1) to zero (N_0) in the PCR step. Table 2 shows the reduction in the number of reactions for the six different 5'/3' combinations, with a proportional increase in the number of expected fragments per lane. In the case of N_0 , only the "heel" portion (see Fig. 5) need provide the sequence for a 3' PCR primer. These latter numbers were statistically derived using 4-cutter sites of sequences from the Expressed Gene Anatomy Database (EGAD) of The Institute for Genomic Research (www.tigr.org) and projected for mRNA complexity of 15,000 unique transcripts. Only 3'-proximal fragments up to 400 bp in size were considered in the statistical analysis since this represents the upper limit of gel resolution. This size, on the other hand, is significantly greater than the mean size of fragments generated by 4-cutters and therefore only a small fraction will be lost by gel exclusion.

From Table 2, it can be seen that strategies III&V are likely candidates for parsing reduction since they yield manageable numbers of both reactions and resolvable fragments. Strategies I, II and IV produce too many reactions, while strategy VI produces a large number of fragments that cannot be readily resolved.

The present invention is not intended to be limited to the preceding embodiments, but rather to encompass all such variations and modifications as come within the scope of the appended claims.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

- (i) APPLICANT: Guilfoyle, Richard A
Guo, Zhen
- 5 (ii) TITLE OF INVENTION: Nucleic Acid Indexing
- (iii) NUMBER OF SEQUENCES: 32
- (iv) CORRESPONDENCE ADDRESS:
 - 10 (A) ADDRESSEE: Quarles & Brady
 - (B) STREET: 1 South Pinckney St.
 - (C) CITY: Madison
 - (D) STATE: WI
 - (E) COUNTRY: US
 - (F) ZIP: 53703
- 15 (v) COMPUTER READABLE FORM:
 - (A) MEDIUM TYPE: Floppy disk
 - (B) COMPUTER: IBM PC compatible
 - (C) OPERATING SYSTEM: PC-DOS/MS-DOS
 - (D) SOFTWARE: PatentIn Release #1.0, Version #1.30
- 20 (vi) CURRENT APPLICATION DATA:
 - (A) APPLICATION NUMBER:
 - (B) FILING DATE:
 - (C) CLASSIFICATION:
- 25 (viii) ATTORNEY/AGENT INFORMATION:
 - (A) NAME: Berson, Bennett J
 - (B) REGISTRATION NUMBER: 37094
 - (C) REFERENCE/DOCKET NUMBER: 960296.94053
- (ix) TELECOMMUNICATION INFORMATION:
 - 30 (A) TELEPHONE: 608-251-5000
 - (B) TELEFAX: 608-251-9166

(2) INFORMATION FOR SEQ ID NO:1:

- (i) SEQUENCE CHARACTERISTICS:
 - 35 (A) LENGTH: 18 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: other nucleic acid
 - (A) DESCRIPTION: /desc = "-21M13 forward primer"
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:
 - 40 TGTAACACGA CGGCCAGT

18

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

- 5 (A) LENGTH: 27 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

- (A) DESCRIPTION: /desc = "end specific
 adaptor (517L)"

10 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

ACATTTTGCT GCCGGTCACT AGTGGTC

27

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:

- 15 (A) LENGTH: 27 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

- 20 (A) DESCRIPTION: /desc = "end specific
 adaptor (560L)"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

ACATTTTGCT GCCGGTCACT AGTGGTA

27

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:

- 25 (A) LENGTH: 27 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

- 30 (A) DESCRIPTION: /desc = "end specific
 adaptor (1567L)"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

ACATTTTGCT GCCGGTCACT AGTGATA

27

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:

- 35 (A) LENGTH: 27 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
 (A) DESCRIPTION: /desc = "end specific
 adaptor (2684L) "

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

5 ACATTTTGCT GCCGGTCACT AGTAGTC

27

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 27 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

10

(ii) MOLECULE TYPE: other nucleic acid
 (A) DESCRIPTION: /desc = "end specific
 adaptor (4459L) "

15 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

ACATTTTGCT GCCGGTCACT AGTGGGC

27

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 27 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

20

(ii) MOLECULE TYPE: other nucleic acid
 (A) DESCRIPTION: /desc = "end specific
 adaptor (4623L) "

25

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

ACATTTTGCT GCCGGTCACT AGTCAAG

27

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 27 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

30

(ii) MOLECULE TYPE: other nucleic acid
 (A) DESCRIPTION: /desc = "end specific
 adaptor (6330L) "

35

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

ACATTTTGCT GCCGGTCACT AGTCAAA

27

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:

- 5 (A) LENGTH: 27 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

- (A) DESCRIPTION: /desc = "end specific
 adaptor (18909L) "

10 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

ACATTTTGCT GCCGGTCACT AGTCGGC

27

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:

- 15 (A) LENGTH: 18 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

- (A) DESCRIPTION: /desc = "M13RevP reverse primer"

20 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

CAGGAAACAG CTATGACC

18

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:

- 25 (A) LENGTH: 27 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

- 30 (A) DESCRIPTION: /desc = "end specific
 adaptor (517R) "

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

GTCCTTTGTC GATACTGGCT AGTGAAG

27

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:

- 35 (A) LENGTH: 27 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

- 40 (A) DESCRIPTION: /desc = "end specific
 adaptor (1576R) "

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

GTCCTTTGTC GATACTGGCT AGTCAGT

27

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:

- 5 (A) LENGTH: 27 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

- 10 (A) DESCRIPTION: /desc = "end specific
adaptor (2684R)"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

GTCCTTTGTC GATACTGGCT AGTCGGA

27

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:

- 15 (A) LENGTH: 27 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

- 20 (A) DESCRIPTION: /desc = "end specific
adaptor (4459R)"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

GTCCTTTGTC GATACTGGCT AGTGGAG

27

25 (2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:

- 30 (A) LENGTH: 27 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

- (A) DESCRIPTION: /desc = "end specific
adaptor (4623R)"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

35 GTCCTTTGTC GATACTGGCT AGTTCCT

27

(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:

- 5 (A) LENGTH: 27 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

- (A) DESCRIPTION: /desc = "end specific
adaptor (6330R)"

10 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

GTCCTTTGTC GATACTGGCT AGTTGAC

27

(2) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:

- 15 (A) LENGTH: 27 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

- 20 (A) DESCRIPTION: /desc = "end specific
adaptor (8848R)"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

GTCCTTTGTC GATACTGGCT AGTTTAG

27

(2) INFORMATION FOR SEQ ID NO:18:

(i) SEQUENCE CHARACTERISTICS:

- 25 (A) LENGTH: 27 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

- 30 (A) DESCRIPTION: /desc = "end specific
adaptor (18909R)"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

GTCCTTTGTC GATACTGGCT AGTGGTG

27

(2) INFORMATION FOR SEQ ID NO:19:

(i) SEQUENCE CHARACTERISTICS:

- 35 (A) LENGTH: 27 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: other nucleic acid
 - (A) DESCRIPTION: /desc = "combinatorial adaptor invading strand for forward primer"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

5 ACATTTTGCT GCCGGTCACT AGTNNNN 27

(2) INFORMATION FOR SEQ ID NO:20:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 27 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: other nucleic acid
 - (A) DESCRIPTION: /desc = "combinatorial adaptor invading strand for reverse primer"

15 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

GTCCTTTGTC GATACTGGCT AGTNNNN 27

(2) INFORMATION FOR SEQ ID NO:21:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 18 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: other nucleic acid
 - (A) DESCRIPTION: /desc = "lambda terminal primer (right end)"

25

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

CGTAACCTGT CGGATCAC 18

(2) INFORMATION FOR SEQ ID NO:22:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 18 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: other nucleic acid
 - (A) DESCRIPTION: /desc = "lambda primer (left end)"

30

35

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

CGCGGGTTTT CGCTATTT 18

(2) INFORMATION FOR SEQ ID NO:23:

- 5 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 26 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "end specific adaptor"
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

10 ACATTTTGCT GCCGGTCACT AGGACC 26

(2) INFORMATION FOR SEQ ID NO:24:

- 15 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 26 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "end specific adaptor"
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:24:

20 ACATTTTGCT GCCGGTCACT AGCGAC 26

(2) INFORMATION FOR SEQ ID NO:25:

- 25 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 26 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "end specific adaptor"
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:25:

30 ACATTTTGCT GCCGGTCACT AGCCGA 26

(2) INFORMATION FOR SEQ ID NO:26:

- 35 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 26 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "end specific adaptor"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:26:

ACATTTTGCT GCCGGTCACT AGGAGA

26

(2) INFORMATION FOR SEQ ID NO:27:

(i) SEQUENCE CHARACTERISTICS:

- 5 (A) LENGTH: 22 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

- 10 (A) DESCRIPTION: /desc = "M13 reverse primer with
NlaIII adaptor"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:27:

CAGGAAACAG CTATGACCCA TG

22

(2) INFORMATION FOR SEQ ID NO:28:

(i) SEQUENCE CHARACTERISTICS:

- 15 (A) LENGTH: 18 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

- 20 (A) DESCRIPTION: /desc = "adaptor strand"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:28:

GTCCTTTGTC GATACTGG

18

(2) INFORMATION FOR SEQ ID NO:29:

(i) SEQUENCE CHARACTERISTICS:

- 25 (A) LENGTH: 27 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

- 30 (A) DESCRIPTION: /desc = "invading and primer strand
for 3'-overhang adaptor"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:29:

NNNNCTGCAT GACCGGCAGC AAAATGT

27

35 (2) INFORMATION FOR SEQ ID NO:30:

(i) SEQUENCE CHARACTERISTICS:

- 40 (A) LENGTH: 18 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "oligonucleotide
complementary to M13 forward primer"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:30:

5 ACATTTTGCT GCCGGTCA

18

(2) INFORMATION FOR SEQ ID NO:31:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 27 base pairs
(B) TYPE: nucleic acid
10 (C) STRANDEDNESS: single
(D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "oligonucleotide
15 complementary to M13 forward primer after
ligation to 3' overhang restriction
fragment end"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:31:

ACATTTTGCT GCCGGTCATG CAGNNNN

27

(2) INFORMATION FOR SEQ ID NO:32:

- 20 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 40 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
- 25 (ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "oligonucleotide
anchor primer for cDNA synthesis and PCR
primer in fragment amplification step"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:32:

30 CAGGGTAGAC GACGCTACGC TTTTTTTTTT TTTTTTTTAT

40

CLAIMS

WE CLAIM:

1. A method for indexing polynucleotides, comprising the steps of:
- 5 (A) combining under base-pairing conditions:
- (i) one or more distinguishable sets of indexing adaptors, each adaptor comprising at least one terminus having a single-stranded region characterized by, in non-overlapping order inward from the end: (a)
- 10 an indexing sequence n bases long and (b) a sequence characteristic of cleavage by a Class II restriction endonuclease, wherein n is an integer and wherein further each set of adaptors comprises one or more indexing sequences, and
- 15 (ii) one or more corresponding polynucleotides, each of which comprises at least one terminus characterized by, in non-overlapping order inward from the end: (a) a region having a sequence characteristic of cleavage by a Class II restriction endonuclease and
- 20 (b) a double-stranded region having on one strand a sequence that base-pairs with an indexing sequence of an adaptor and on the other strand a sequence complementary thereto;
- (B) base-pairing at least one adaptor terminus and at
- 25 least one corresponding polynucleotide terminus to form at least one strand-displaced structure wherein the indexing sequence of the single-stranded region of the adaptor terminus is base-paired with the sequence that base-pairs with an indexing sequence of the terminus of the
- 30 corresponding polynucleotide, and the complementary sequence on the other strand of the polynucleotide terminus is displaced from base-pairing thereto;
- (C) for each adaptor set, distinguishing the corresponding polynucleotides that form strand-displaced
- 35 structures, thereby indexing the polynucleotides by their base-pairing to the distinguishable sets of adaptors.

2. A method according to claim 1, wherein n is 1 to 4.

3. A method according to claim 2 wherein n is 2, 3 or 4.

5 4. A method according to claim 1, wherein the indexing adaptors in all the sets of adaptors together comprise all possible sequences of A, T, G and C n bases long.

5. A method according to claim 1, further comprising
10 the step of ligating, in a strand-displaced structure, the end of the adaptor strand comprising the indexing sequence to the end of the abutting strand of the double-stranded region of the corresponding polynucleotide.

6. A method according to claim 1, wherein one or more
15 adaptors further comprise a sequence for amplification.

7. A method according to claim 1, wherein strand-displaced structures are formed at both ends of at least one corresponding polynucleotide.

8. A method according to claim 7, wherein the adaptor
20 at each terminus of at least one corresponding polynucleotide further comprises at least one sequence for amplification by PCR.

9. A method according to claim 8, further comprising the step of ligating, in the strand-displaced structure of
25 each terminus of at least one polynucleotide, the end of the adaptor strand comprising the indexing sequence to the end of the abutting strand of the double-stranded region of the corresponding polynucleotide.

10. A method according to claim 9, further comprising the step of amplifying by PCR corresponding polynucleotides having adaptor strands ligated to each terminus, using the adaptor sequences for amplification.

5 11. A method according to claim 6, further comprising the step of amplifying at least one polynucleotide using primers defined by the sequence for amplification.

12. A method according to claim 1, wherein the sequence characteristic of cleavage by a Class II
10 restriction endonuclease has a 3'-terminated single-stranded region.

13. A method according to claim 1, wherein the sequence characteristic of cleavage by a Class II
15 restriction endonuclease has a 5'-terminated single-stranded region.

14. A method according to claim 1, wherein the sequence characteristic of cleavage by a Class II restriction endonuclease has a blunt end.

15. A method for characterizing a population of polynucleotides, comprising the steps of:

(A) combining under base-pairing conditions:

5 (i) one or more distinguishable sets of indexing adaptors, each adaptor comprising at least one terminus having a single-stranded region characterized by, in non-overlapping order inward from the end: (a) an indexing sequence n bases long and (b) a sequence characteristic of cleavage by a Class II restriction
10 endonuclease, wherein n is an integer and wherein further each set of adaptors comprises one or more indexing sequences, and

(ii) a population of polynucleotides that includes one or more corresponding polynucleotides,
15 each of which comprises at least one terminus characterized by, in non-overlapping order inward from the end: (a) a region having a sequence characteristic of cleavage by a Class II restriction endonuclease and (b) a double-stranded region having on one strand a
20 sequence that base-pairs with an indexing sequence of an adaptor and on the other strand a sequence complementary thereto;

(B) base-pairing at least one adaptor terminus to at least one corresponding polynucleotide terminus to form at
25 least one strand-displaced structure wherein the indexing sequence of the single-stranded region of the adaptor terminus is base-paired with the sequence that base-pairs with an indexing sequence of the terminus of the corresponding polynucleotide, and the complementary
30 sequence on the other strand of the polynucleotide terminus is displaced from base-pairing thereto;

(C) for each adaptor set, determining one or more corresponding polynucleotides that form strand-displaced structures, or the absence thereof;

35 (D) characterizing the population of polynucleotides by the corresponding polynucleotides that form strand-displaced structures, or the absence thereof.

16. A method according to claim 15, wherein n is 1 to 5.

17. A method according to claim 15, wherein the population is a population of cDNAs or other
5 polynucleotides representative of mRNAs.

18. A method according to claim 17, wherein the characterization is indicative of gene expression in a sample from which the population was derived.

19. A method according to claim 15, wherein the
10 population is a population of genomic DNAs or polynucleotides representative of genomic DNAs.

20. A method according to claim 19, wherein the characterization comprises characterizing mutations or the absence thereof in: (a) the sequence characteristic of
15 cleavage by a Class II restriction endonuclease; (b) the indexing sequence or both (a) and (b) of one or more corresponding polynucleotides in the population.

21. A method according to claim 20, wherein the absence or presence of a mutation thus characterized in one
20 or more corresponding polynucleotides is diagnostic of a potential to develop one or more diseases, or of one or more diseases.

22. A method according to claim 15, further comprising the step of ligating, in a strand-displaced
25 structure, the end of the adaptor-strand comprising the indexing sequence to the end of the abutting strand of the double-stranded region of the corresponding polynucleotide.

23. A method according to claim 15, wherein one or more adaptors further comprise a sequence for
30 amplification.

24. A method according to claim 15, wherein strand-displaced structures are formed at both ends of at least one corresponding polynucleotide.

25. A method according to claim 24, wherein the
5 adaptor at each terminus of at least one corresponding polynucleotide further comprises at least one sequence for amplification by PCR.

26. A method according to claim 25, further
comprising the step of ligating, in the strand-displaced
10 structure of each terminus of at least one polynucleotide, the end of the adaptor strand comprising the indexing sequence to the end of the abutting strand of the double-stranded region of the corresponding polynucleotide.

27. A method according to claim 26, further
15 comprising the step of amplifying by PCR corresponding polynucleotides having adaptor strands ligated to each terminus, using the adaptor sequences for amplification either as a primer or as a primer binding site.

28. A method according to claim 15, further
20 comprising, for at least one adaptor set, resolving from one another at least two corresponding polynucleotides that form strand-displaced structures.

29. A method according to claim 28, wherein the population is a population of cDNAs or other polynucleotides representative of mRNAs in a sample, the size and the quantity of the separated corresponding
5 polynucleotides is determined, each separated corresponding polynucleotide is identified by the indexing sequence and the Class II restriction endonuclease characteristic sequence of the adaptor set with which it formed a strand-displaced structure and by its size, and the quantities of
10 the thus identified corresponding polynucleotides for at least two adaptor sets provides a profile of gene expression in the source from which the cDNA was derived.

30. A method according to claim 28, wherein corresponding polynucleotides for at least one set of
15 adaptors are resolved by size by electrophoresis.

31. A method according to claim 29, wherein the sequences characteristic of a Class II restriction endonuclease and the indexing sequences of the adaptor sets together subdivide the cDNAs or other polynucleotides
20 representative of the mRNAs into sets of corresponding polynucleotides in each of which corresponding polynucleotides can be individually differentiated by electrophoresis.

32. A method for comparing mRNA in two or more
25 samples, comprising the steps of claim 29 to generate a profile of gene expression of a first sample and, independently, a profile of gene expression in a second sample and comparing the profiles of the first and second samples.

33. A set of adaptors, each adaptor comprising at least one terminus having a single-stranded region characterized by, in non-overlapping order inward from the end: (a) an indexing sequence n bases long and (b) a
5 sequence characteristic of cleavage by a Class II restriction endonuclease, wherein n is an integer, the set of adaptors comprising adaptors with at least two of the possible indexing sequences n bases long.

34. A set of adaptors according to claim 33, wherein
10 n is 1, 2, 3, 4 or 5.

35. A set of adaptors according to claim 34, wherein n is 2, 3 or 4.

36. A set of adaptors according to claim 33, wherein the bases are A, C, G and T.

15 37. A set of adaptors according to claim 33, wherein the bases are selected from the group consisting of A, C, G, T and modified bases.

38. A set of adaptors according to claim 33, comprising adaptors with all possible indexing sequences of
20 A, C, G or T n bases long.

39. A set of adaptors according to claim 38, wherein n is 1, 2, 3, 4 or 5.

40. A set of adaptors according to claim 39, wherein n is 2, 3 or 4.

25 41. A set of adaptors according to claim 33, comprising adaptors with all possible indexing sequences n bases long, the bases being one or more of the group consisting of A, C, G, T and modified bases.

42. A method for amplifying a polynucleotide, comprising the steps of:

(A) combining under base-pairing conditions:

5 (1) an adaptor comprising: (a) at least one terminus having a single-stranded region characterized by, in non-overlapping order inward from the end, (i) a sequence n bases long for base-pairing to one or more polynucleotides, where n is an integer and (ii) a sequence characteristic of cleavage by a Class II
10 restriction endonuclease, and (b) a region comprising a sequence for amplifying a polynucleotide, and

(2) a polynucleotide comprising at least one terminus characterized by, in non-overlapping order inward from the end, (a) a region having a sequence
15 characteristic of cleavage by a Class II restriction endonuclease and (b) a double-stranded region having on one strand a sequence that base-pairs with the single-stranded adaptor sequence for base-pairing with a polynucleotide, and on the other strand a sequence
20 complementary thereto;

(B) base-pairing the single-stranded adaptor sequence for base-pairing to one or more polynucleotides with the polynucleotide sequence that base-pairs therewith and displacing the complementary sequence on the other strand
25 of the polynucleotide terminus;

(C) ligating the end of the adaptor strand comprising the indexing sequence to the end of the abutting strand of the double-stranded region of the polynucleotide; and

(D) amplifying the polynucleotide using the adaptor
30 sequence for amplifying a polynucleotide.

43. A method according to claim 42, wherein the sequence for amplifying a polynucleotide is selected from the group consisting of a primer for elongating a template by a DNA polymerase and a sequence complementary to a
35 primer for elongating a template by a DNA polymerase.

44. A method according to 43, wherein adaptors are ligated at both ends of the polynucleotide and exponential amplification of the polynucleotide is carried out by PCR using primers defined by the sequences of the adaptors for
5 amplifying a polynucleotide.

45. A method according to claim 42, wherein more than two adaptors each having a different sequence for base-pairing with a polynucleotide are used to base pair specifically to different polynucleotides in a population
10 of polynucleotides.

46. A method according to claim 42, wherein an adaptor is ligated to each end of the polynucleotides and exponential amplification of the polynucleotides is carried out by PCR using primers defined by the sequences for
15 amplification of the adaptors.

47. A method according to claim 46, wherein the sequences of the adaptors for base-pairing to one or more polynucleotides together comprise all sequences of A, C, G, and T n bases long.

48. A method for isolating a polynucleotide, comprising the steps of:

(A) combining under base-pairing conditions:

5 (1) an adaptor comprising: (a) at least one terminus having a single-stranded region characterized by, in non-overlapping order inward from the end, (i) a sequence n bases long for base-pairing to one or more polynucleotides, where n is an integer and (ii) a sequence characteristic of cleavage by a Class II
10 restriction endonuclease, and (b) a region comprising a sequence for amplifying a polynucleotide, and

(2) a polynucleotide comprising at least one terminus characterized by, in non-overlapping order inward from the end, (a) a region having a sequence
15 characteristic of cleavage by a Class II restriction endonuclease and (b) a double-stranded region having on one strand a sequence that base-pairs with the single-stranded adaptor sequence for base-pairing with a polynucleotide, and on the other strand a sequence
20 complementary thereto;

(B) base-pairing the single-stranded adaptor sequence for base-pairing to a polynucleotide with the polynucleotide sequence that base-pairs therewith and displacing the complementary sequence on the other strand
25 of the polynucleotide terminus;

(C) ligating the end of the adaptor strand comprising the indexing sequence to the end of the abutting strand of the double-stranded region of the polynucleotide;

(D) amplifying the polynucleotide using the adaptor
30 sequence for amplifying a polynucleotide; and

(E) isolating the amplified polynucleotide.

49. A method according to claim 48, wherein the sequence for amplifying a polynucleotide is selected from the group consisting of a primer for elongating a template
35 by a DNA polymerase and a sequence complementary to a primer for elongating a template by a DNA polymerase.

50. A method according to 49, wherein adaptors are ligated at both ends of the polynucleotide and exponential amplification of the polynucleotide is carried out by PCR using primers defined by the sequences of the adaptors for
5 amplifying a polynucleotide.

51. A method according to claim 48, wherein two or more adaptors each having a different sequence for base-pairing with a polynucleotide are used to base pair specifically to two or more different polynucleotides in a
10 population of polynucleotides.

52. A method according to claim 51, wherein an adaptor is ligated to each end of the polynucleotides and exponential amplification of the polynucleotides is carried out by PCR using primers defined by the sequences of the
15 adaptors for amplifying a polynucleotide.

53. A method according to claim 48, wherein the amplified polynucleotide is resolved from other polynucleotides by gel electrophoresis and then eluted from the gel.

20 54. A method according to claim 48, further comprising the step of cloning the amplified polynucleotide.

55. One or more kits together comprising a plurality of adaptors, wherein:

(A) each adaptor comprises at least one terminus having a single-stranded region characterized by, in non-
5 overlapping order inward from the end, (a) an indexing sequence n bases long wherein n is an integer, and (b) a sequence characteristic of cleavage by a Class II restriction endonuclease, and

(B) the plurality of adaptors comprises for each
10 given sequence characteristic of cleavage by a Class II restriction endonuclease at least one adaptor having at least one specific indexing sequence.

56. A kit or kits according to claim 55, wherein the sequence characteristic of cleavage by a Class II
15 restriction endonuclease is selected from the group consisting of a sequence having a 5' overhang and a sequence having a 3' overhang.

57. A kit or kits according to claim 56, wherein the Class II restriction endonuclease is selected from the
20 group consisting of BclI, NotI, DpnII, BamHI, HindIII, AvrII, ApaLI, KpnI, SphI, NsiI, and SacI.

58. A kit or kits according to claim 56, wherein n is 1, 2 or 3.

59. A kit or kits according to claim 56, wherein the
25 plurality of adaptors comprises a set of adaptors with indexing sequences that base-pair with each sequence n bases long of A, C, G, and T, where n is an integer.

60. A kit or kits according to claim 59, wherein the base-pairing specificity of each base of the adaptor indexing sequences is selected from the group consisting of A, C, G, T, Py, Pu and N, wherein Py denotes base pairing
5 to A and G, Pu denotes base-pairing to C and T and N denotes base-pairing to A, C, G and T.

61. A kit or kits according to claim 60, wherein the bases of the indexing sequences are selected from the group consisting of A, C, T, G and X, where X is a nucleoside
10 other than A, C, T or G that can form specific base-pairs with A, C, G or T in DNA.

62. A kit or kits according to 61, wherein X contains the modified base 3'-nitropyrrole or the modified base 5'-nitroindole.

15 63. A kit or kits according to claim 61, wherein the plurality of adaptors comprises a set of adaptors having each indexing sequence n bases long of A, C, G and T, where n is an integer.

64. A method for indexing polynucleotides, comprising the steps of:

(A) combining under base-pairing conditions:

5 (i) one or more distinguishable sets of indexing adaptors, each adaptor comprising a sequence for amplification and at least one terminus having a single-stranded region characterized by, in non-overlapping order inward from the end: (a) an indexing sequence n bases long and (b) a sequence
10 characteristic of cleavage by a Class II restriction endonuclease, wherein n is an integer and wherein further each set of adaptors comprises one or more indexing sequences, and

15 (ii) one or more corresponding polynucleotides, each of which comprises at least one terminus characterized by, in non-overlapping order inward from the end: (a) a region having a sequence characteristic of cleavage by a Class II restriction endonuclease and (b) a double-stranded region having on one strand a
20 sequence that base-pairs with an indexing sequence of an adaptor and on the other strand a sequence complementary thereto;

(B) base-pairing at least one adaptor terminus and at least one corresponding polynucleotide terminus to form at
25 least one strand-displaced structure wherein the indexing sequence of the single-stranded region of the adaptor terminus is base-paired with the sequence that base-pairs with an indexing sequence of the terminus of the corresponding polynucleotide, and the complementary
30 sequence on the other strand of the polynucleotide terminus is displaced from base-pairing thereto;

(C) for each adaptor set, amplifying the corresponding polynucleotides that form strand-displaced structures using the sequence for amplification; and

35 (D) distinguishing the amplified corresponding polynucleotides thereby indexing the polynucleotides by their base-pairing to the distinguishable sets of adaptors.

65. A method according to claim 64, wherein n is 1 to 4.

66. A method according to claim 65 wherein n is 2, 3 or 4.

5 67. A method according to claim 64, wherein the indexing adaptors in all the sets of adaptors together comprise all possible sequences of A, T, G and C n bases long.

68. A method according to claim 64, wherein the
10 sequence for amplification is selected from a group consisting of a PCR primer, a T3 promoter, a T7 promoter, an SP6 promoter, and a sequence complementary to any of the same.

69. A method according to claim 64, wherein the
15 amplification is an exponential amplification.

70. A method according to claim 64, further comprising the step of ligating, in a strand-displaced structure, the end of the adaptor strand comprising the indexing sequence to the end of the abutting strand of the
20 double-stranded region of the corresponding polynucleotide.

71. A method according to claim 70, wherein strand-displaced structures are formed at both ends of at least one corresponding polynucleotide.

72. A method according to claim 71, wherein the
25 sequence for amplification is a PCR primer.

73. A method according to claim 64, wherein the sequence characteristic of cleavage by a Class II restriction endonuclease has a 3'-terminated single-stranded region.

74. A method according to claim 64, wherein the sequence characteristic of cleavage by a Class II restriction endonuclease has a 5'-terminated single-stranded region.

- 5 75. A method according to claim 64, wherein the sequence characteristic of cleavage by a Class II restriction endonuclease has a blunt end.

76. A method for characterizing a population of polynucleotides, comprising the steps of:

(A) combining under base-pairing conditions:

5 (i) one or more distinguishable sets of indexing adaptors, each adaptor comprising a sequence for amplification and at least one terminus having a single-stranded region characterized by, in non-overlapping order inward from the end: (a) an indexing sequence n bases long and (b) a sequence
10 characteristic of cleavage by a Class II restriction endonuclease, wherein n is an integer and wherein further each set of adaptors comprises one or more indexing sequences, and

15 (ii) a population of polynucleotides that includes one or more corresponding polynucleotides, each of which comprises at least one terminus characterized by, in non-overlapping order inward from the end: (a) a region having a sequence characteristic of cleavage by a Class II restriction endonuclease and
20 (b) a double-stranded region having on one strand a sequence that base-pairs with an indexing sequence of an adaptor and on the other strand a sequence complementary thereto;

(B) base-pairing at least one adaptor terminus to at
25 least one corresponding polynucleotide terminus to form at least one strand-displaced structure wherein the indexing sequence of the single-stranded region of the adaptor terminus is base-paired with the sequence that base-pairs with an indexing sequence of the terminus of the
30 corresponding polynucleotide, and the complementary sequence on the other strand of the polynucleotide terminus is displaced from base-pairing thereto;

(C) for each adaptor set, amplifying corresponding polynucleotides that form strand-displaced structures using
35 the sequence for amplification;

(D) determining one or more amplified corresponding polynucleotides or the absence thereof; and

(E) characterizing the population of polynucleotides by the amplified corresponding polynucleotides that form strand-displaced structures using the sequence for amplification, or the absence thereof.

5 77. A method according to claim 76, wherein n is 1 to 5.

78. A method according to claim 76, wherein the population is a population of cDNAs or other polynucleotides representative of mRNAs.

10 79. A method according to claim 78, wherein the characterization is indicative of gene expression in a sample from which the population was derived.

80. A method according to claim 76, wherein the population is a population of genomic DNAs or
15 polynucleotides representative of genomic DNAs.

81. A method according to claim 80, wherein the characterization comprises characterizing mutations or the absence thereof in: (a) the sequence characteristic of cleavage by a Class II restriction endonuclease; (b) the
20 indexing sequence or both (a) and (b) of one or more corresponding polynucleotides in the population.

82. A method according to claim 81, wherein the absence or presence of a mutation thus characterized in one or more corresponding polynucleotides is diagnostic of a
25 potential to develop one or more diseases, or of one or more diseases.

83. A method according to claim 76, further comprising the step of ligating, in a strand-displaced structure, the end of the adaptor-strand comprising the indexing sequence to the end of the abutting strand of the double-stranded region of the corresponding polynucleotide.

84. A method according to claim 83, wherein strand-displaced structures are formed at both ends of at least one corresponding polynucleotide.

85. A method according to claim 84, wherein the sequence for amplification is a PCR primer.

86. A method according to claim 85, further comprising the step of amplifying by PCR corresponding polynucleotides having adaptor strands ligated to each terminus, using the sequences for amplification either as a primer or as a primer binding site.

87. A method according to claim 76, further comprising, for at least one adaptor set, resolving from one another at least two corresponding polynucleotides that form strand-displaced structures.

88. A method according to claim 87, wherein the population is a population of cDNAs or other polynucleotides representative of mRNAs in a sample, the size and the quantity of the separated corresponding polynucleotides is determined, each separated corresponding polynucleotide is identified by the indexing sequence and the Class II restriction endonuclease characteristic sequence of the adaptor set with which it formed a strand-displaced structure and by its size, and the quantities of the thus identified corresponding polynucleotides for at least two adaptor sets provides a profile of gene expression in the source from which the cDNA was derived.

89. A method according to claim 88, wherein corresponding polynucleotides for at least one set of adaptors are resolved by size by electrophoresis.

90. A method according to claim 88, wherein the
5 sequences characteristic of a Class II restriction endonuclease and the indexing sequences of the adaptor sets together subdivide the cDNAs or other polynucleotides representative of the mRNAs into sets of corresponding polynucleotides in each of which corresponding
10 polynucleotides can be individually differentiated by electrophoresis.

91. A method for comparing mRNA in two or more samples, comprising the steps of claim 88 to generate a profile of gene expression of a first sample and,
15 independently, a profile of gene expression in a second sample and comparing the profiles of the first and second samples.

92. A method according to claim 76, wherein the sequence for amplification is selected from a group
20 consisting of a PCR primer, a T3 promoter, a T7 promoter, an SP6 promoter, and a sequence complementary to same.

1/5

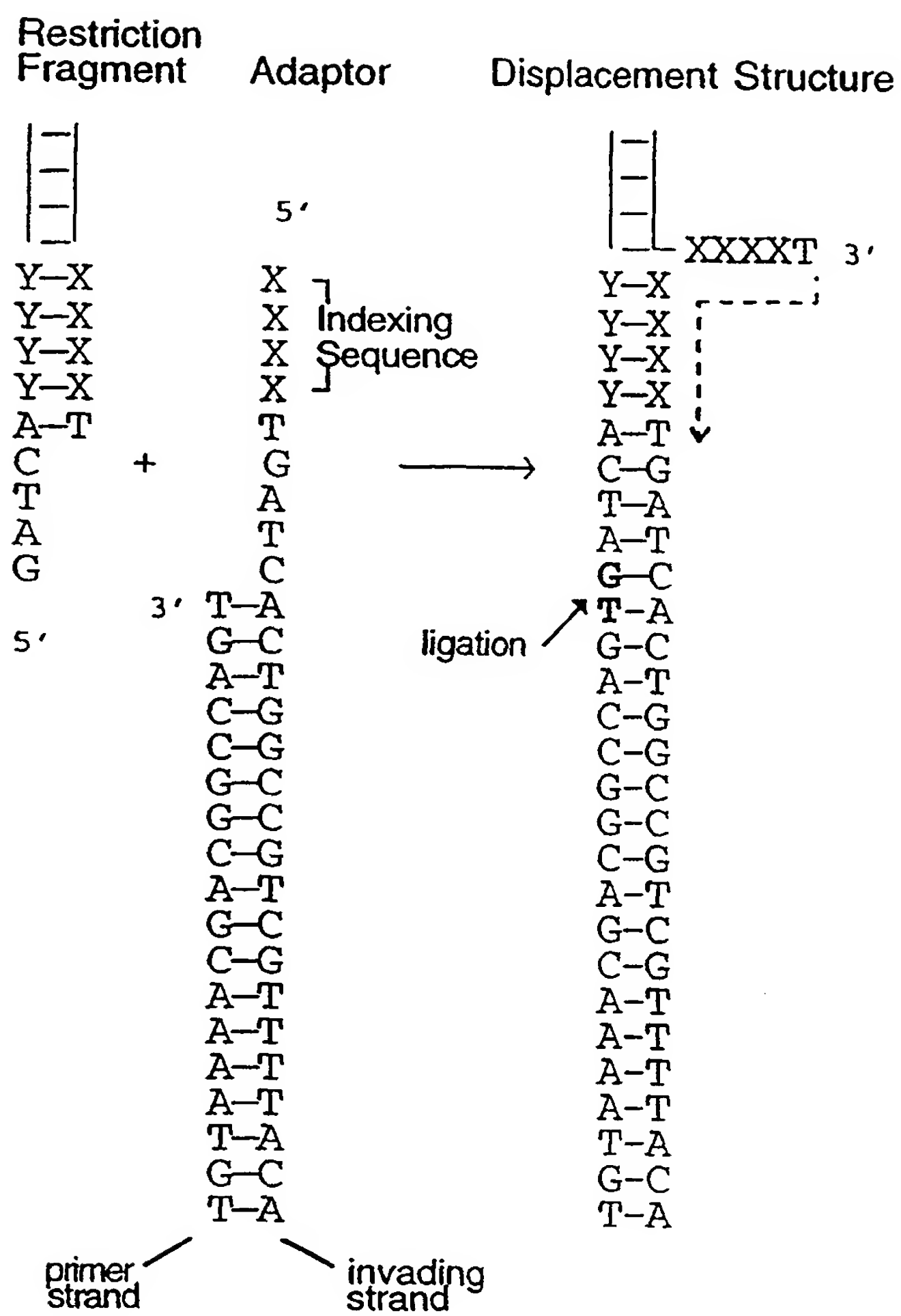


FIG 1

2/5

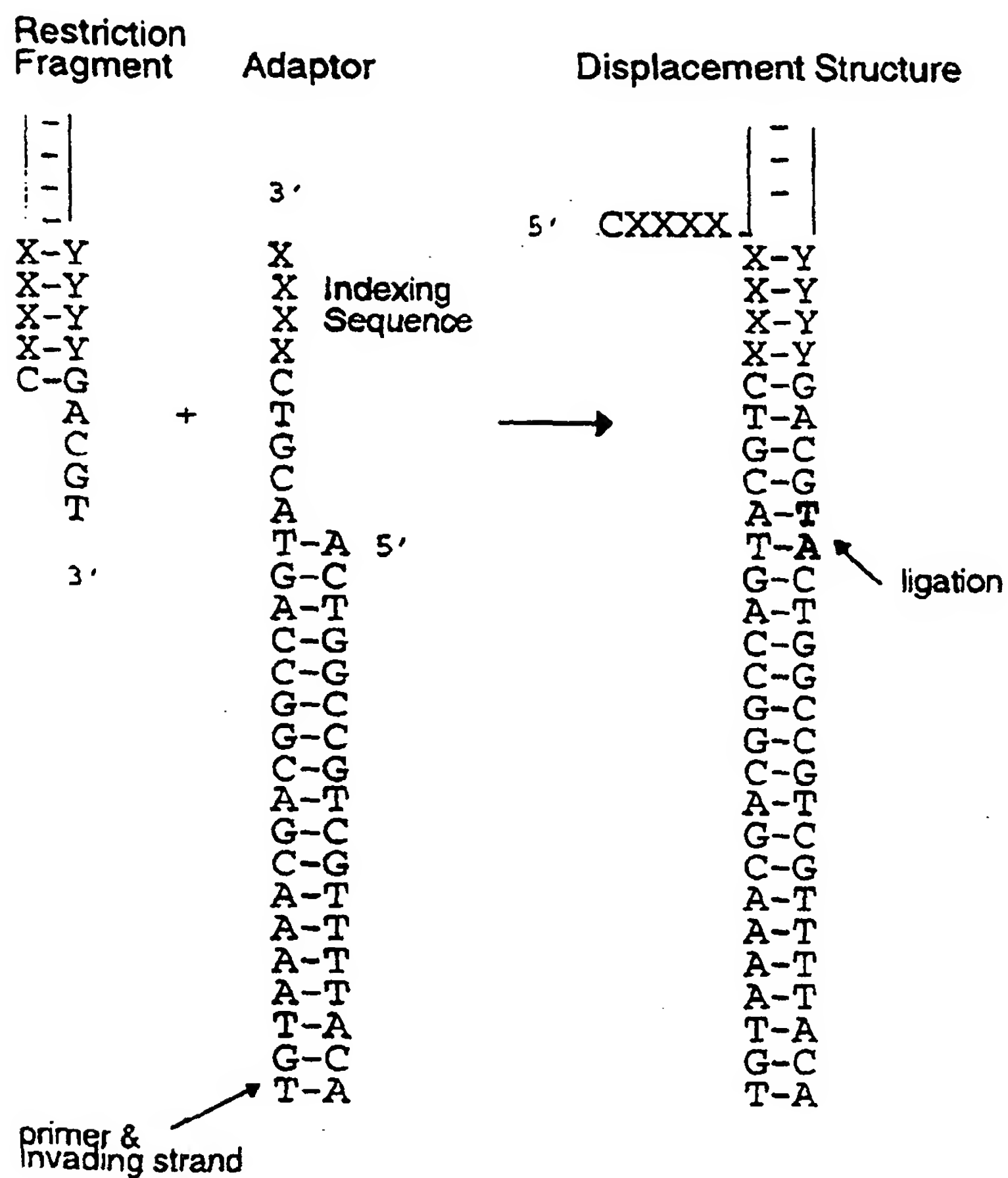


FIG 2

RECTIFIED SHEET (RULE 91)
ISA/EP

3/5

FIG 3A *END-SPECIFIC ADAPTORS*LEFT *w/forward primer*

-21M13	5'	tgtaaaacgacggccagt
517L	3'	ACATTTTGCTGCCGGTCACTAGTGGTC
560L		ACATTTTGCTGCCGGTCACTAGTGGTA
1567L		ACATTTTGCTGCCGGTCACTAGTGATA
2684L		ACATTTTGCTGCCGGTCACTAGTAGTC
4459L		ACATTTTGCTGCCGGTCACTAGTGGGC
4623L		ACATTTTGCTGCCGGTCACTAGTCAAG
6330L		ACATTTTGCTGCCGGTCACTAGTCAAA
18909L		ACATTTTGCTGCCGGTCACTAGTCGGC

RIGHT *w/reverse primer*

M13RevP	5'	caggaaacagctatgacc
517R	3'	GTCCTTTGTCGATACTGGCTAGTGAAG
1576R		GTCCTTTGTCGATACTGGCTAGTCAGT
2684R		GTCCTTTGTCGATACTGGCTAGTCGGA
4459R		GTCCTTTGTCGATACTGGCTAGTGGAG
4623R		GTCCTTTGTCGATACTGGCTAGTTCCT
6330R		GTCCTTTGTCGATACTGGCTAGTTGAC
8848R		GTCCTTTGTCGATACTGGCTAGTTTAG
18909R		GTCCTTTGTCGATACTGGCTAGTGGTG

FIG 3B *COMBINATORIAL ADAPTORS w/forward or reverse primer*

Combo-FP	5'	tgtaaaacgacggccagt
	3'	ACATTTTGCTGCCGGTCACTAGTNNNN
Combo-RP	5'	caggaaacagctatgacc
	3'	GTCCTTTGTCGATACTGGCTAGTNNNN

SUBSTITUTE SHEET (RULE 26)

DpnII adaptors w/forward primer

```
5'      tgtaaaacgacggccagt
3'      ACATTTTGCTGCCGGTCACTAGGACC
        ACATTTTGCTGCCGGTCACTAGCGAC
        ACATTTTGCTGCCGGTCACTAGCCGA
        ACATTTTGCTGCCGGTCACTAGGAGA
```

NlaIII adaptor w/reverse primer

```
5'      CAGGAAACAGCTATGACCCATG
3'      GTCCTTTGTCGATACTGG
```

FIG 4

5/5

Sau3AI: 5' ∇ GATC
CTAG_A

tgtaaaacgacggccagt + GATCTTTT----- IL-2
ACATTTTGCTGCCGGTCACTAGAAAA AAAA-----

↓ T4 DNA Ligase

M13 forward →
tgtaaaacgacggccagtGATCTTTT-----//-----
ACATTTTGCTGCCGGTCACTAGAAAA└-----//-----
A
A
A
A 3' ← N₁N₂(dt)₁₈ heel
(AT)

200 = 26 + 142 (-8) + 40

Sau3AI indexing adaptors:

tgtaaaacgacggccagt	
ACATTTTGCTGCCGGTCACTAG GACC	IS-1
CGAC	IS-2
CCGA	IS-3
GAGA	IS-4
GA CT	IS-5
AGTC	IS-6
TATA	IS-7
ATAT	IS-8
AAAA	IS-9 (IL-2)

FIG 5

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 98/04819

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12Q1/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WONG D M ET AL: "BRANCH CAPTURE REACTIONS: DISPLACERS DERIVED FROM ASYMMETRIC PCR" NUCLEIC ACIDS RESEARCH, vol. 19, no. 9, 11 May 1991, pages 2251-2259, XP000204316 see whole doc., esp. figure 1, p.2254 ---	1-92
Y	EP 0 735 144 A (JAPAN RES DEV CORP) 2 October 1996 see the whole document --- -/--	1-92

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

*** Special categories of cited documents :**

"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier document but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
"&" document member of the same patent family

Date of the actual completion of the international search

2 September 1998

Date of mailing of the international search report

30/09/1998

Name and mailing address of the ISA
European Patent Office, P.B. 5818 Patentlaan 2
NL - 2280 HV Rijswijk
Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,
Fax: (+31-70) 340-3016

Authorized officer

Müller, F

INTERNATIONAL SEARCH REPORT

International Application No.
PCT/US 98/04819

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	<p>KATO K: "DESCRIPTION OF THE ENTIRE MRNA POPULATION BY A 3' END CDNA FRAGMENT GENERATED BY CLASS IIS RESTRICTION ENZYMES"</p> <p>NUCLEIC ACIDS RESEARCH, vol. 23, no. 18, September 1995, pages 3685-3690, XP002008304 see the whole document</p> <p style="text-align: center;">---</p>	1-92
A	<p>UNRAU P. & DEUGAU K.V.: "Non-cloning amplification of specific DNA fragments from whole genomic DNA digests using DNA 'indexers'"</p> <p>GENE, vol. 145, - 1994 pages 163-169, XP002054436 see the whole document</p> <p style="text-align: center;">---</p>	1-92
P,X	<p>GUILFOYLE R.A. ET AL.,:</p> <p>"Ligation-mediated PCR amplification of specific fragments from class-II restriction endonuclease total digest"</p> <p>NUCLEIC ACIDS RESEARCH, vol. 25, no. 9, - 1 May 1997 pages 1854-1858, XP002076198 see the whole document</p> <p style="text-align: center;">-----</p>	1-92

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/US 98/04819

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP 0735144 A	02-10-1996	JP 2763277 B	11-06-1998
		JP 9028399 A	04-02-1997
		JP 2763278 B	11-06-1998
		JP 8322598 A	10-12-1996
		AU 692685 B	11-06-1998
		AU 5031196 A	10-10-1996
		US 5707807 A	13-01-1998
